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Lactogenic Hormone Content of the AP of the Pigeon.*

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It is quite generally accepted at the present time that the factor in the anterior pituitary which stimulates the proliferation of the pigeon crop glands and the initiation and maintenance of milk secretion in mammals is the same.[1,2] As a consequence the pigeon assay method for the determination of the amount of lactogenic hormone in the anterior pituitary of experimental animals has been preferred to the mammalian lactation test due to the much smaller amount of hormone and the short time required to obtain a positive response. This is true, especially, of the intradermal "micro" assay method.[1,3]

In spite of the general use of the pigeon as an assay animal, only a few qualitative observations have been reported concerning the lactogen content of the pigeon anterior pituitary. Riddle and Schooley[4] observed crop weight increases in pigeons following the implantation of pituitary glands from mature female and immature common pigeons.

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 889.

1 Bergman, A. J., Meites, J., and Turner, C. W., *Endocrinology*, 1940, **26**, 716.

2 Bergman, A. J., and Turner, C. W., *J. Dairy Sci.*, 1940, **23**, 1229.

3 Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707.

4 Riddle, O., and Schooley, J. P., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 1610.

Leblond and Noble[5] reported that the implantation of 5 mixed male and female pigeon pituitaries intradermally over the crop glands of pigeons gave definite proliferation which they graded between +3 to 4. Later Schooley and Riddle[6] showed that the pituitaries of male and female pigeons which had been incubating eggs would cause an increase in crop gland weight when implanted or injected into pigeons.

It seemed of interest, therefore, to determine the lactogen content of the anterior pituitary of the type of pigeon long used in our assays and compare it with the content in one type of large domestic pigeon, the White King.

Procedure. Normal male and female common and White King pigeons were sacrificed and the pituitaries removed, weighed and stored at a freezing temperature until assayed. For the assays, the pituitary glands were macerated, suspended in distilled water, and injected in a constant volume intradermally over the crop gland of common pigeons weighing 260-340 g. Both the minimum-micro technic[1] and the Reece-Turner[3,7] method of assay were employed. Previous study indicated that these assay methods are reproducible within a range of about ± 10%.[1,3]

Results. As in mammals, the pituitary of the male pigeon contains less lactogenic hormone than do the females. This is true in spite of the fact that the male pigeon aids the female in incubating the eggs and in feeding the squabs. The pituitary of the male White King as well as of the common pigeon by both methods of comparison contain only ½ to ⅓ as much lactogen as the female (Table I).

The common female pigeon, although considerably lighter in body weight than the Silver King, contains as much or more lactogen per pituitary. Per 100 g body weight, the pituitary of the Silver King contains only ½ to ⅔ as much lactogen as the common pigeon.

Discussion. The presence of a factor in the pituitaries of birds which appears to be identical with the factor in the mammalian pituitary concerned with the initiation of lactation is of interest phylogenetically. In the chicken, this factor seems to be related to the condition of broodiness;[8,9] in the pigeon with both broodiness and the secretion of a crop "milk" as well. In the pigeon, the proliferation of the crop glands is initiated with the onset of the incubation period[10,11] and reaches a climax at the time of hatching to feed the young. From these observations, it appears that the primitive activity of this factor of the pituitary was concerned with the brooding action of birds and in the preparation of the crop glands to secrete a satisfactory food; *i.e.*, pigeon crop milk for the newly hatched young.

In the evolution of the mammalian types,

TABLE I.
Lactogenic Hormone Content of the Pigeon Pituitary.

Group	Sex	No. of birds	Body wt, g	Pituitary wt, mg	Avg I.U. of lactogen: Per pituitary	Per mg pituitary	Per 100 g body wt
White King	F	8	486	4.29	.09*	.02	.02
" "	M	3	505	4.40	.03*	.01	.005
Common Pigeons	F	11	303	3.15	.13*	.04	.04
" "	F	8	276	4.32	.09†	.02	.03
" "	M	16	302	3.92	.03†	.01	.01

* Assayed by the minimum-micro technic.[1] (160 units = 1 I.U.)
† Assayed by the Reece-Turner method.[7] (22.2 units = 1 I.U.)

5 Leblond, C. P., and Noble, G. K., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 517.

6 Schooley, J. P., and Riddle, O., *Am. J. Anat.*, 1938, **62**, 313.

7 Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 266, 1937.

8 Burrows, W. H., and Byerly, T. C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 841.

9 Byerly, T. C., and Burrows, W. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 844.

10 Beams, H. W., and Meyer, R. K., *Physiol. Zool.*, 1931, **4**, 486.

the egg-laying monotremes, such as the duckbill platypus and the Australian ant-eater, would require a factor comparable to that present in birds to stimulate the act of incubation and thus provide for the hatching of the young.[12] It would seem reasonable that this same factor would play a role in the initiation of lactation at the close of the incubation period in many ways comparable to the relation of this factor in stimulating the gradual proliferation of a crop secretion to be utilized by the newly hatched squab.

The male pigeon takes part in the incubation of the eggs and in the feeding of the squabs. It is strange, therefore, that the inactive male pigeon pituitary contains definitely less lactogenic hormone than the female. In this respect it is quite comparable to the sex difference in the lactogenic content of mammals.[13]

The mechanism by which the pigeon pituitary is activated to the production of lactogen and the incubation of the eggs is not known. In the male, Patel[11] has indicated an association with the incubating female and the act of sitting on the eggs to be necessary for the formation of crop milk. Further, castration in one incubation cycle did not influence crop milk production by the male in that cycle but prevented "milk" production in succeeding periods.

The mechanism by which the lactogenic hormone in birds is stimulated to increased production in order to produce broodiness and crop milk formation and in the primitive mammals, the egg-laying monotremes, broodiness and milk secretion may be a sensory nervous stimulation comparable to the influence of light upon the gonadotropins of the pituitary. Whether the estrogens and androgens play roles in mediating the effect requires further investigation.

Summary. The lactogen content of the anterior pituitary of two types of pigeons, the common and the White King, were compared in both sexes. As in mammals, the pituitaries of the male pigeons contained only ½ to ⅓ as much lactogen as the female by all methods of comparison even though the male pigeon aids in the incubation of the eggs and in feeding the squabs.

The common female pigeon, although considerably lighter in body weight than the White King, contains as much or more lactogen per pituitary. Per 100 g body weight, the pituitary of the White King contains only ½ to ⅔ as much lactogen as the common pigeon.

11 Patel, M. D., *Physiol. Zool.*, 1936, **9**, 129.

12 Turner, C. W., *The Comparative Anatomy of the Mammary Glands*, Pub. Univ. Coop. Store, Columbia, Mo., 1939.

13 Hurst, V., and Turner, C. W., *Endocrinology*, 1943, **31**, 334.

14200

Inactivation of Estrone in Normal Male Rabbits.

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The metabolism of estrogens in rabbits has been the subject of several conflicting reports. Smith and Smith[1] recovered less than 10% of the injected estrogens in the urine of these animals; Frank and his collaborators[2] found practically none in the blood after 30 minutes. The original observations were confirmed by Pincus and Zahl[3,4] and their findings implicated the uterus as an agent in the conversion of estrone to estriol, and the ovaries

1 Smith, G. S., and Smith, O. W., *Am. J. Physiol.*, 1931, **98**, 578.

2 Frank, R. T., Goldberger, M. A., and Spielman, F., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 1229.

3 Pincus, G., *Cold Spring Harbor Symposia on Quant. Biol.*, 1937, **5**, 44.

4 Pincus, G., and Zahl, R. A., *J. Gen. Physiol.*, 1937, **20**, 879.

in the conversion of estradiol to estrone. Subsequent experiments showed that the ovaries and uterus are not necessary for the conversion of *a*-estradiol to estrone and *b*-estradiol.[5,6] Using the *in vitro* technic Heller[7] and Heller and Heller[8] reported that rabbit liver can inactivate estrogens in a manner similar to rat liver and that the function is not altered by the pregnant state.

Numerous observations indicate that the liver is a specific site of inactivation of estrogens in rats, dogs, and human beings; these investigations have been summarized elsewhere.[9,10] As the liver exerts so profound an effect on the metabolism of estrogens in these species, it is important that this function be considered in any study on the inactivation or interconversion of estrogens *in vivo*. Moreover, the hepatic estrogen-inactivating function is readily affected by nutritional deficiency,[10] even of moderate degree. Thus the experiments to be described were undertaken to ascertain whether or not the liver of the intact rabbit, like that of the other species mentioned, also inactivates estrogen. The method previously used in rats[11,12] has been applied to the rabbit and confirms that in this species too the liver alone is capable of inactivating estrone. Male animals were employed to eliminate any possible interfering activity by the uterus or ovaries.

Methods. The young male rabbits used in this experiment varied in weight between 1200 and 2500 g, and were between 3 and 4 months old. In each of 3 of these animals 2 weighed pellets of estrone were implanted subcutaneously. In each of a second group of 3 animals 2 similar pellets were implanted in the spleen. A third group of 3 animals served as controls. The diet consisted of a standard brand of rabbit pellets.* The weight of each animal was recorded weekly and all showed a continuous gain. The experiment was terminated 91 days after the pellets were inserted. After nembutal anesthesia and exsanguination the testicles were dissected, cleaned of all adherent structures, and weighed immediately on a damped balance. The testicles were fixed in Bouin's solution together with the seminal vesicles, prostate and epididymis. The usual methods were employed for dehydrating, embedding in paraffin, sectioning and staining selected blocks of these tissues.

The experimental data are presented in Table I. It is evident that the weights of the testicles of the animals with the pellets in the spleen are of the same order of magnitude as the controls, while the testicles of the group implanted subcutaneously weigh only about one-sixth as much. Variations in the weights of the animals and in the amount of estrone absorbed were not sufficient to influence the above result.

The microscopic sections of the testicles of the animals with the pellets in the spleens show no significant histologic changes from the normal controls. There is pronounced alteration of the histologic pattern of the testicles of the group implanted subcutaneously; the spermatic tubules are shrunken and are lined by several layers of large vacuolated cells that have replaced entirely the normally active spermatogenic cells. The lamina propria is fibrous and thickened. This histologic pattern is very similar to that found after experimental cryptorchidism in the rabbit,[13] and in normal male rats implanted with estrone pellets in the subcutaneous tissues for 42 days.[12]

The microscopic sections of the prostate and seminal vesicles of the group with the pellets in the spleen are almost indistinguishable from the normal controls. A slight interstitial edema accompanied by a scattering of

5 Fish, W. R., and Dorfman, R. I., *J. Biol. Chem.*, 1941, **140**, xl; 1942, **143**, 15.

6 Heard, R. D. H., Bauld, W. S., and Hoffman, M. M., *J. Biol. Chem.*, 1941, **141**, 709.

7 Heller, C. G., *Endocrinology*, 1940, **26**, 619.

8 Heller, C. G., and Heller, E. J., *Endocrinology*, 1943, **32**, 64.

9 Doisy, E. A., Thayer, S. A., and Van Bruggen, J. T., *Federation Proceedings*, 1942, **1**, 202.

10 Biskind, M. S., and Biskind, G. R., *Endocrinology*, 1942, **31**, 109.

11 Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

12 Biskind, G. R., *Med. Surg. Tributes to Harold Brunn*, Univ. Calif. Press, 1942, p. 41.

* Globe A-1 Wonder Rabbit Pellets, Pillsbury Flour Mills Co., Los Angeles.

13 Biskind, G. R., and Glick, David, *Arch. Path.*, 1937, **23**, 363.

TABLE I.
Estrone Pellets Implanted for 91 Days in the Spleen or in the Subcutaneous Tissues of Male Rabbits: Effect on Testicles; Comparison with Normal Controls.

Animal No.	Location of pellet	Body wt		Wt of testes		Amt estrone absorbed, mg
		Beginning, g	End of exp., g	Right, g	Left, g	
6	—	1850	3370	2.84	2.63	—
7	—	1200	2925	2.35	2.12	—
8	—	2400	3600	2.85	2.75	—
	Avg	1817	3278	2.68	2.50	
9	Subcu.	2500	3750	0.45	0.47	1 pellet lost
10	"	1850	3045	0.43	0.47	4.1
11	"	1700	3075	0.44	0.44	3.0
	Avg	2017	3290	0.44	0.46	3.6
13	Spleen	1800	3000	2.38	2.65	4.0
15	"	2450	3300	2.66	2.70	4.9
16	"	2300	3600	2.62	2.71	4.3
	Avg	2217	3300	2.55	2.69	4.4

lymphocytes and phagocytic mononuclear cells is the only histologic deviation from the normal. The epithelial elements of these organs are similar in these two groups. The atrophic condition of the prostate and seminal vesicles of the animals implanted subcutaneously is readily distinguished from the above. Accompanying the shrunken epithelial elements of these organs is a diffuse scattering of inflammatory cells in the acini and interstitial tissues, with edema and fibrosis of the latter.

Discussion. In previous experiments of this type, performed on rats, a control group of animals was employed to exclude the possibility that the spleen may alter in some manner the absorbed estrogen before it is inactivated by the liver. In this type of control, the pellet was placed in the spleen; then that organ was transplanted into the overlying abdominal wall. After an interval to permit the development of vascular adhesions, the splenic vessels were ligated, thus excluding the spleen from the portal system and forcing the absorbed hormone into the systemic circulation. The hormone under investigation always exerted its specific action under these conditions. The uniformity of results in the previous experiments indicated that the spleen played a passive part as the site of absorption; therefore that control group was omitted in this experiment.

A possible explanation of the slight inflammatory reaction in the prostate and seminal vesicles of the group with the pellets in the spleen is that for a short period immediately after implantation of the pellet some of the hormone escapes inactivation in the liver, as happens in female rats.[11] During this time the organs may have been altered by the hormone with the production of an inflammatory reaction that has persisted long after the epithelial elements have returned to normal.

The absence of any specific action of the absorbed estrone in the animals with pellets in the spleen indicates that the compound is completely inactivated in the liver. Even though estrogens are excreted with bile and may enter into an entero-hepatic circulation, as shown by Cantarow *et al.*,[14] there was still no opportunity in our experiment for the absorbed hormone to be influenced by the genital organs before it reached the liver. Hence, one may conclude that the liver is capable of inactivating estrone without the intermediation of other organs, and that this function takes place in the rabbit as well as in other species previously mentioned.

Summary. The liver of normal male rabbits has the ability to inactivate estrone as shown by the presence of normal testicles and genital apparatus in a group of three animals examined 91 days after the implantation of estrone pellets in the spleens. Alterations in the structure of the testicles in rabbits with subcutaneously implanted pellets corresponded to

[14] Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walking, A. A., *Endocrinology*, 1943, **32**, 368.

the changes found in similarly treated male rats, and to the changes produced by experimental cryptorchidism in rabbits.

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Lactation Activity, Chemical Composition, and *in vitro* Metabolism of Rat Mammary Tissue.

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During work on the metabolic effect of lactogenic hormone one of us observed that mammary tissue from a rat in full lactation consumed *in vitro* 2.7 ± 0.3 cmm of oxygen per mg of dry matter per hour. Mammary tissue from a rat that had suckled only one young, weaned 8 days before the test, consumed *in vitro* only 0.7 ± 0.2 cubic millimeter of oxygen per mg of dry matter per hour.

We have repeated and extended these *in vitro* measurements of the metabolic rate of mammary tissue because the work offered an opportunity to connect metabolic rate with "activity," a term often used rather vaguely.[1] The relation of metabolic rate to the amount of "active" tissue has a sound meaning only when the activity is defined and measured independently of the metabolic rate itself.* Lactation provides such an independent definition af activity. During the trials described here we noted that both lactation activity and metabolic rate are related to the nitrogen and water content of the mammary tissue.

Method. The rats used were bred in our colony from the Long-Evans strain and kept on our ordinary colony diet. On the day of the respiration trial they were killed by a blow on the head. Immediately the thorax was opened; the mammary gland was placed in oxygenated balanced ion glucose solution containing phosphate buffer;[2] and parts of the gland, excised with a forceps, were placed in buffered balanced-ion glucose solution and subjected to Warburg's standard procedure of micro respiration trial in a water-bath kept at 37°C. The time between killing the rat and installing the manometers in the waterbath averaged to 18 ± 1 minute and did not exceed 27 minutes. The amount of tissue used in each respiration chamber was measured by determining the nitrogen in the sample after the trial by the micro Kjeldahl method. In extra samples of the same tissue, the nitrogen : dry matter ratio and the water content were determined.

Results. Table I summarizes the results of these measurements with mammary tissue taken on the 20th day of pregnancy and tissue taken on the 21st day of lactation. The number of fetuses per pregnant rat ranged from 9 to 11. Each of the 8 lactating rats included in the means suckled 6 young. This is the size to which each larger litter in our colony is reduced at birth.

The lactating mammary tissue contained less than half as much dry matter as the mammary tissue from the pregnant rats. The mammary tissue of a rat that had suckled only 3 young contained, on the 21st day of lactation, 26% of dry matter—a figure higher than that for any rat with 6 young. The mammary dry-matter content of a rat with only 2 young was as high as 35%. Lactation, therefore, increases the water content of the mammary gland. The nitrogen content of the dry mammary tissue had the same trend as the water content of the fresh gland. The dry matter of the lactating mammary glands averaged over 7 times as much nitrogen per

1 Benedict, F. G., *Vital Energetics*, 1938, 199.

* Otherwise one relates just two different expressions for only one variable.

2 Kleiber, M., and Cole, H. H., *Am. J. Physiol.*, 1939, **125**, 747.

TABLE I.
Lactation Activity, Composition, and Metabolic Rate of Rat Mammary Tissue.

Condition of rat at time of microrespiration trial with mammary tissue		20th day of pregnancy	21st day of lactation
No. of rats included in means		5	8
Mean No. of fetuses per rat		10 ± 0.5	
Mean No. of young suckled per rat			6 ± 0
Dry-matter % of mammary tissue	%	59 ± 5	23 ± 1
Nitrogen % in dry mammary tissue	%	1.3 ± 0.5	9.5 ± 0.3
Oxygen consumption *in vitro* per hour:			
Per mg of moist tissue	mm^3	0.5 ± 0.1	0.6 ± 0.1
" " " dry tissue	mm^3	0.9 ± 0.2	2.9 ± 0.3
" " " nitrogen	mm^3	88 ± 17	31 ± 3

unit weight as the dry nonlactating mammary tissue. The 2 rats with less than 6 young apiece again confirm the general trend; the nitrogen content of their mammary dry matter was 7.8 and 5.7% respectively for the tissue from the rat with 3 and that with 2 offspring. Lactation thus increases the protein content of the dry matter in the mammary gland.

The metabolic rate *in vitro* per mg of fresh tissue is within the range of the standard deviation—the same for lactating and nonlactating mammary tissue. The ordinary Warburg quotient Q_{O_2} (cmm of oxygen consumed per hour per mg dry tissue) is, however, 3 times as high for the lactating mammary tissue as for the mammary tissue taken from the pregnant rats. This increasing effect of lactation on the metabolic rate per unit dry tissue is statistically highly significant. The metabolic rate per unit nitrogen is, in contrast to that per unit dry matter, only one-third as high in the lactating tissue as in the tissue from the pregnant rat.

The results of our micro respiration trials lead, accordingly, to the puzzling conclusion that lactation does not affect the metabolic

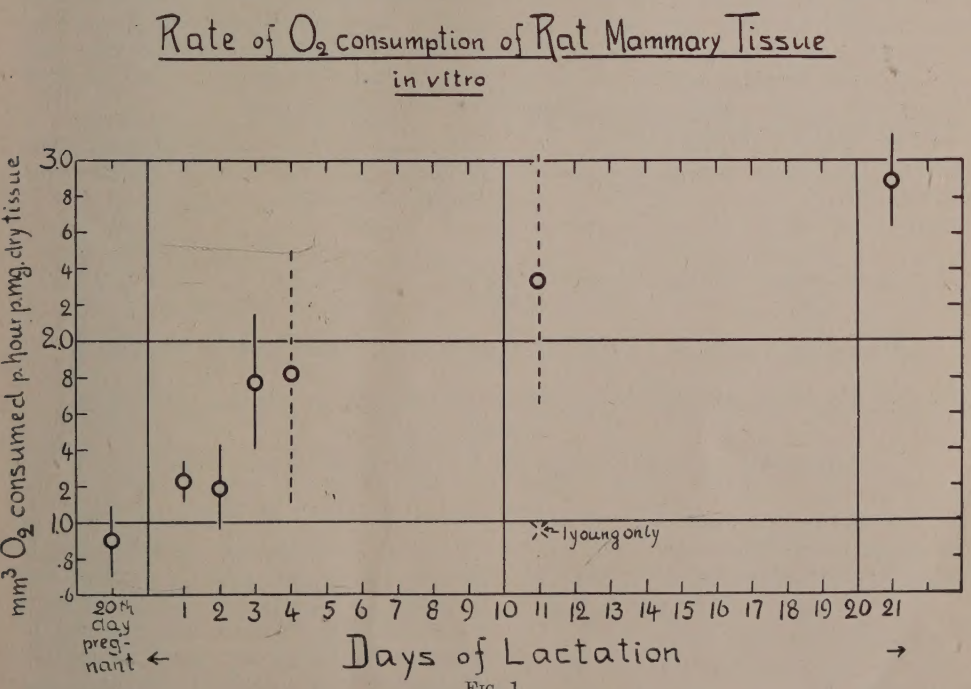

FIG. 1.
Rate of O_2 consumption of rat mammary tissue.

rate of the mammary gland, and yet increases it or decreases it, depending on whether one means the metabolic rate per mg of fresh tissue, per mg dry matter, or per mg nitrogen.

The nitrogen content, usually taken as "the most serviceable measure for the active protoplasmic tissue,"[1] may be misleading as such a basis for the metabolic rate of the mammary gland, because much of the nitrogen in the lactating tissue might be inert milk protein or a precursor of this substance.

Fig. 1 shows the trend of *in vitro* metabolic rate per unit of dry matter for the duration of lactation. The standard deviation of the means is indicated by the vertical lines connected to the circles. In two instances (namely, on the 4th and 11th day of lactation) when we had only the results from one rat each, the broken vertical line indicates the extent of the standard deviation for a single measurement as calculated from the data on the 3rd day.

The metabolic rate *in vitro* per unit of dry tissue increases up to the 21st day of lactation. The rate of milk production in rats in terms of milk energy per day also rises up to this time, according to Brody and Nisbet[3] (see Fig. C, page 13). Fig. 1, accordingly, indicates a positive correlation between metabolic rate per unit of dry matter and lactation activity through the lactation period. The low Warburg quotient at the 11th day (marked with a cross in Fig. 1) resulted from the mammary tissue of a rat that suckled only one young. This result also confirms the rule that lactation intensity and metabolic rate per unit of mammary dry matter are positively correlated.

Summary. Mammary tissue of rats at the end of pregnancy and at the height of lactation was analyzed for dry matter and nitrogen content, and the metabolic rate of this tissue was measured *in vitro*.

Lactation increased the water content of the mammary tissue and the protein content of the mammary dry matter.

Lactation did not affect the metabolic rate per unit of fresh tissue *in vitro;* it increased the metabolic rate per unit of dry matter and decreased the metabolic rate per unit of nitrogen in the tissue.

Observations were also made on rats that suckled only 1-3 instead of 6 young; and measurements were taken on rats with 6 young at earlier stages of lactation before full milk production was reached. These indicate a positive correlation between the intensity of lactation and the magnitude of the effect that lactation has on the composition and metabolic rate of mammary tissue.

The authors are glad to acknowledge that Robert E. Smith helped them effectively with the microrespiration trials.

[3] Brody, S., and Nisbet, Ruth, *Missouri Research Bul.*, 285, 1938.

14202 P

Effect of Estradiol on Urinary Excretion of Ascorbic Acid in the Dog.

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An investigation was made of the effect of the estrogen, estradiol-benzoate (Progynon-B, Schering†), on the urinary excretion of ascorbic acid by the dog, an animal capable of synthesizing ascorbic acid. Studies were made of the influence of estradiol on the 24-hour excretion and on the mechanism of ascorbic acid clearance. This was done to determine if changes in total excretion might be due to alteration of the normal renal mechanism, which consists of filtration at the glomerulus

* Commonwealth Research Fellow.

† Supplied by courtesy of Dr. Max Gilbert, Schering Corp.

and active tubular reabsorption.[1]

Methods. Control values of the maximal tubular reabsorptive capacity (Tm) for ascorbic acid of 3 female dogs were determined by repeated clearance studies within 34 days of the beginning of treatment. This was done by constant intravenous infusion of ascorbic acid and of creatinine, the clearance of the latter measuring glomerular filtration rate in the dog. Ascorbic acid was infused at a rate sufficient to give load levels in excess of that needed for tubular saturation. The clearance values of 3 or 4 successive urine collection periods were averaged in computing the data. The dogs were hydrated prior to the clearance to maintain adequate urine flows.

The creatinine determinations were made by the method of Folin and Wu,[2] after the plasmas had been precipitated by the $CdSO_4$ method.[3] The ascorbic acid of both plasma and urine was determined by a modification of the method of Mindlin and Butler.[4]

Two of the dogs were kept in metabolism cages so that 24-hour collections of urine could be made. Deterioration of the ascorbic acid was minimized by the addition of sulphuric acid to the collection vessels.

Daily injections of 1.66 mg of estradiol-benzoate in sesame oil were given intramuscularly for periods of 10 to 14 days. During this time, 24-hour collections of urine were continued and further clearance determinations of the ascorbic acid Tm were made. To allow the plasma levels to return to normal values the urine collections were resumed 18 to 24 hours after infusion of the ascorbic acid. Fasting plasma levels of ascorbic acid were determined before and during estradiol treatment.

Results. Although a renal threshold exists, some ascorbic acid is lost in the urine at low plasma levels. Dog A had an average daily control excretion of 83.0 mg (range: 64-89 mg); dog B, 112 mg (range: 101-125 mg). The control fasting plasma levels of ascorbic acid in dog A averaged 0.49 mg%; in dog B, 0.43 mg%. The average control ascorbic acid Tm values (mg/min) were as follows: dog A, 0.581; dog B, 0.531; dog C, 0.560.

The effects of the injection of estradiol became apparent in 3 or 4 days by a rise in the daily excretion of ascorbic acid, by a decrease in tubular reabsorption, and by a rise in the ascorbic acid clearance/creatinine clearance ratio. The 24-hour excretion rose to an average of 112.0 mg (range: 97-129) in dog

TABLE I.
Effect of Estradiol on Clearance of Ascorbic Acid in the Dog.

		Dog A: 19 kg .087 mg Progynon/kg		Dog B: 18 kg .092 mg Progynon/kg		Dog C: 13.5 kg .123 mg Progynon/kg	
		Control	Treated	Control	Treated	Control	Treated
No. of clearance exper.*		7	6	7	6	6	7
Load:	range:	1.16-2.35	1.14-2.36	1.43-2.69	1.66-2.88	1.42-1.94	1.08-1.65
	(mg/min) avg:	1.67	1.71	2.04	2.12	1.61	1.47
Tm:	range:	.489-.661	.408-.537	.410-.628	.063-.351	.461-.671	.150-.420
	(mg/min) avg:	.581	.480	.531	.224	.560	.306
$\frac{C_{aa}}{C_{cr}}$†	range:	.540-.725	.600-.773	.638-.776	.812-.974	.608-.695	.667-.890
	avg:	.636	.704	.733	.900	.659	.790

* Each clearance experiment consists of 3 or 4 urine collection periods.

† $\frac{C_{aa}}{C_{cr}} = \frac{\text{Ascorbic acid clearance}}{\text{Creatinine clearance}}$

[1] Sherry, S., Friedman, G. J., Paley, K., Berkman, J., and Ralli, E. P., *Am. J. Physiol.*, 1940, **130**, 276.

[2] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

[3] Fujita, A., and Iwatake, D., *Biochem. Z.*, 1931, **242**, 43.

[4] Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

A and to 186 mg (range: 154-243) in dog B. The fasting plasma levels of ascorbic acid decreased to 0.32 mg% in dog A and to 0.34 mg% in dog B. This was accompanied by an average decrease in reabsorptive Tm of 17.5% in dog A, 58% in dog B, and 45.3% in dog C. The clearance ratio rose 11, 23, and 20% respectively.

Conclusions. Estradiol increases the clearance of ascorbic acid in the dog by reducing tubular reabsorption, the clearance approaching that of creatinine in some cases. As a result, the fasting plasma levels of ascorbic acid fall during the treatment.

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A Test Paper for the Rapid Estimation of the Level of Sulfonamide in Serum.

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The reaction of Ehrlich's reagent, *p*-dimethylaminobenzaldehyde, with aromatic amines in acid solution has been made the basis of a colorimetric determination of sulfonamides by Werner[1] and by Morris.[2] Fuller[3] has employed this substance in test papers for estimating the level of sulfonamide in laked blood after precipitation of proteins with an acid reagent, comparing the yellow color produced with standard color strips. The present paper describes the preparation of acid-containing test papers with which the level of sulfonamide can be estimated directly in serum without the addition of precipitating or acidifying reagents.

One gram of *p*-dimethylaminobenzaldehyde (straw-colored material is usable) is dissolved in 2 cc of concentrated hydrochloric acid and to the solution is added 0.8 cc of syrupy phosphoric acid (sp. gr. about 1.7) and water to make 100 cc. Large pieces of a good grade of absorbent paper (such as is used in making litmus paper) are soaked in the solution and immediately hung up to drain. When superficially dry, the papers are stored in the dark in covered glass jars for from 5 to 10 days, during which time most of the hydrochloric acid evaporates. The edges of the paper, which are usually yellow, are then trimmed off and the paper is cut into small strips and preserved in suitable vials. The strips are almost colorless, but become discolored if they come in contact with the skin at any time during preparation or use. The phosphoric acid remaining in the papers is sufficient to give a pH of approximately 3 when they are moistened with serum.

Estimation of the sulfonamide level is made by spreading evenly the serum to be tested over one end of an impregnated strip by means of a fine dropper, care being taken that unabsorbed fluid does not remain. In this way, the serum is spread over the paper by the movement of the dropper rather than by the capillarity of the fibers, and the reagents are consequently not leached out of areas which are oversaturated. Within 10 seconds a maximum development of color occurs. To avoid translucence of the paper interfering with the reading, the wet strip is held close to a white background or placed on white paper or white porcelain. The color is then judged in comparison with a graded series of dry standard color strips. The color ranges from a pale violet with normal human serum to a bright yellow with serum containing 15-20 mg sulfadiazine per 100 cc. A comparison of values obtained by the test-paper method with those secured by the method of Bratton and Marshall[4] are set forth in Table I.

1 Werner, A. E. A., *Lancet*, 1939, **1**, 18.

2 Morris, C. J., *Biochem. J.*, 1941, **35**, 952.

3 Fuller, A. T., *Lancet*, 1942, **1**, 760.

4 Bratton, A. C., Marshall, E. K., Babbitt, D., and Hendrickson, A. R., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE I.
Comparative Sulfadiazine Estimations.

Serum sample No.	Method of Bratton and Marshall mg%	Estimation by test papers — Observer* A mg%	B mg%	C mg%	D mg%
1	0.0	0	0	0	0
2	0.0	0	0	0	0
3	0.0	0	0	0	0
4	5.7	6	7	4	4
5	4.3	4	5	4	3
6	5.1	5	6	4	4
7	14.2	14	11	12	11
8	9.3	10	9	8	11
9	9.5	10	—	—	—
10	6.2	6	7	10	11
11	6.4	6	8	8	8
12	3.6	4	6	6	5
13	7.3	8	9	6	8
14	0.0	0	0	0	0
15	7.2	6	4	4	4
16	7.5	8	—	—	—
17	7.1	8	6	6	6
18	8.0	8	8	8	8
19	6.2	6	9	4	8
20	14.2	14	13	12	12
21	7.8	8	5	8	11
22	6.8	8	8	6	10
23	8.3	8	10	8	11
24	5.5	4	7	4	4
25	7.3	6	7	6	8
26	0.0	0	0	0	0
27	4.8	4	6	8	4
28	10.3	10	11	12	10
29	10.0	10	10	8	10
30	11.2	10	10	8	12
31	9.7	—	8	12	12

* A—Experienced observer. B, C, D—Inexperienced observers.

Satisfactory color standards may be made by coloring paper strips with either water colors or soluble dyes. Suitable graded concentrations of picric acid in combination with Orange G to furnish the weak reddish component have been satisfactory. Standards made by selection of appropriate tints from samples of colored construction papers should prove satisfactory. The colors are calibrated by comparison with test strips moistened under the usual conditions with human sera containing known amounts of sulfonamide as determined in serum by standard methods. A single color standard is suitable for estimation of sulfapyridine, sulfathiazole or sulfadiazine; for sulfanilamide the values read from this standard should be divided by 1.5.

The standard does not apply to urine and body fluids containing variable amounts of protein. The colors can be grouped in intensities corresponding to 0-3, 3-6, 6-9, 9-12 and over 12 mg of sulfadiazine per 100 cc or in other convenient groupings. After some practice readings may be obtained which agree within 1 mg (plus or minus) with determinations made by the method of Bratton and Marshall. In any event, in the hands of a worker experienced in the clinical interpretation of sulfonamide levels, serum concentrations should be readily classifiable into groupings such as "zero," "subtherapeutic," "therapeutic" and "toxic." The limits of concentration for any group will depend, as in the method of Bratton and Marshall, on the type of sulfonamide administered. Sulfonamide values for whole blood are not directly transferable to serum values, since the latter are usually higher for the same blood sample.

The reaction on the test papers is given by free sulfonamides and other primary aromatic

amines including *p*-aminobenzoic acid, *i.e.*, by the same compounds that give color in the method of Bratton and Marshall.[4] Urea in pathological concentrations yields significant amounts of color with Ehrlich's reagent. Pyrrols and indoles, however, do not react with the reagent at the acidity attained in the test. Moderate hemolysis in the serum seems not to interfere seriously with color matching. The chief bars to accuracy appear at the present time to be (a) the possibility that the test papers or the standards may prove to be unstable under certain conditions of storage or exposure and (b) the fact that since the intensity of the color is influenced by protein and the test is empirically standardized for normal protein content of serum, alterations of the proteins in pathological sera might lead to inaccuracies. The first difficulty can be removed in part by keeping test papers in a cool place away from direct light and moisture. The other limitations as yet have not been adequately explored. Results of attempts to replace the phosphoric acid by oxalic or citric acids in the preparation of test papers indicate that modification in this direction is possible.

14204

The Chemotherapeutic Effect of Esters of Penicillin.*

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In a recent communication[1] the preparation of esters of penicillin was reported. Although aliphatic esters show no bacteriostatic action *in vitro*, it was shown that they are highly effective chemotherapeutic agents in experimental infections due to hemolytic streptococci. Evidence was presented to show that they are effective *in vivo* both by the subcutaneous and by the oral route of administration. This activity is undoubtedly due to the hydrolysis of the esters with a slow liberation of active penicillin.

In this paper experiments will be described which confirm and extend the previous observations.

Materials and Methods. The penicillin employed throughout the study was prepared by the chloroform method.[2] The free acid was esterified with diazoethane or diazo-n-butane prepared from the corresponding derivatives of nitroso-urea. Remaining acidic fractions were separated from the ester by alkaline phosphate extraction.† Unless otherwise specified, the esters were dissolved in absolute alcohol before use and the solutions diluted with four volumes of propylene glycol.

In all experiments 15-hour blood broth cultures of a highly virulent strain of Group A hemolytic streptococci (strain C203Mv) were used. Mice were infected by the intraperitoneal route with 1 cc of varying dilutions of culture. Treatment was carried out by either the subcutaneous or the oral route of administration. All experiments were controlled with a series of untreated animals.

Subcutaneous Administration. In the first experiments mice infected with 1 cc of 10^{-3}, 10^{-4}, and 10^{-5} dilutions of hemolytic streptococci were treated with ethyl esters of peni-

* This work has been supported in part by a grant from the John and Mary R. Markle Foundation.

1 Meyer, K., Hobby, G. L., and Chaffee, E., *Science*, 1943, **97**, 205.

2 Meyer, K., *et al.*, *Science*, 1942, **96**, 20.

† Oily fractions removed from both the ethyl and butyl esters contained ethers and addition products of the diazo compounds, probably pyrazoline derivatives. These oily fractions were only weakly active *in vivo* and were locally irritating.

TABLE I.
Subcutaneous Administration of Ethyl Esters of Penicillin in Divided Dosage.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. survived (>7 days)
1.4-4.5	10^{-3}	14		14
	10^{-4}	15	1	14
	10^{-5}	15	1	14
Total		44	2 (4.5%)	42 (95.5%)
0.6	10^{-3}	3	2	1
	10^{-4}	2	1	1
	10^{-5}	3	1	2
Total		8	4 (50%)	4 (50%)
Controls	10^{-7}	12	12 (100%)	

TABLE II.
Subcutaneous Administration of Ethyl Esters of Penicillin in Single Dosage.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. survived (>7 days)
2.0	10^{-4}	3		3
	10^{-5}	3		3
	10^{-6}	3		3
	10^{-7}	3		3
Total		12		12 (100%)
Controls	10^{-7}	10	10 (100%)	

cillin given by the subcutaneous route. Treatment was started one-half hour after infection. Two to 4 injections of 0.1-1.0 mg were given on the first day and one to 2 injections of the same amount on the second day.

As shown in Table I, amounts equal to 1.5 mg of ethyl ester or more were adequate to protect 42 of 44 mice against 10^{-3}, 10^{-4}, and 10^{-5} dilutions of hemolytic streptococci (approximately 100-10,000 lethal doses). With 0.6 mg there was little protection.

In a subsequent experiment a small series of mice similarly infected with hemolytic streptococci were treated with a single subcutaneous injection of 2 mg of ethyl ester given one-half hour after infection.

As shown in Table II, this single injection was adequate to give complete protection against 10^{-4} dilutions of hemolytic streptococci (1000 lethal doses).

The chemotherapeutic effect of n-butyl esters was studied in a larger series of animals. Both unfractionated and more highly purified fractionated preparations of the ester were tested. Varying amounts of the unfractionated and fractionated butyl esters, ranging from 0.5 to 2.5 mg, were given in a single subcutaneous injection one-half hour after infection.

As shown in Table III, 1.5 mg of unfractionated butyl ester was adequate to give complete protection against 10^{-4} to 10^{-6} dilutions of hemolytic streptococci. With 0.5 mg only slight protection was obtained.

When more highly purified fractionated n-butyl esters were used, as shown in Table IV, 0.4 mg was adequate for prolonged life or complete protection of 100% of infected mice. With 0.2 mg there was only partial protection.

Oral Administration. Mice infected with hemolytic streptococci were also treated by the oral route of administration.

Table V shows a small series of which 13 of 16 mice were protected against 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions of hemolytic streptococci by a single dose of 5.0 mg of ethyl ester given by mouth. The remaining 3 mice showed prolonged life. With smaller amounts of ethyl ester, there was no protection.

Similar experiments were carried out in

TABLE III.
Subcutaneous Administration of Unfractionated n-Butyl Esters of Penicillin in Single Dose.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. prolonged life (2-7 days)	No. survived (>7 days)
2.5	10^{-4}	3			3
	10^{-5}	3			3
	10^{-6}	3			3
1.5	10^{-4}	6			6
	10^{-5}	6			6
	10^{-6}	6			6
Total		27			27 (100%)
0.5	10^{-4}	7	7		
	10^{-5}	7		1	6
	10^{-6}	7	2	2	3
Total		21	9 (42.9%)	3 (14.2%)	9 (42.9%)
Controls	10^{-7}	9	9 (100%)		

TABLE IV.
Subcutaneous Administration of Fractionated n-Butyl Esters of Penicillin in Single Dosage.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. prolonged life (2-7 days)	No. survived (>7 days)
0.5-1.5	10^{-4}	15		1	14
	10^{-5}	15			15
	10^{-6}	14		4	10
0.4	10^{-4}	4		1	3
	10^{-5}	4			4
	10^{-6}	3			3
Total		55		6 (10.9%)	49 (89.1%)
0.2	10^{-4}	4	2		2
	10^{-5}	4	1	1	2
	10^{-6}	4	1		3
Total		12	4 (33.3%)	1 (8.3%)	7 (58.4%)
Controls	10^{-6}	12	12		
	10^{-7}	12	12		
Total		24	24 (100%)		

TABLE V.
Oral Administration of Ethyl Esters of Penicillin in Single Dosage.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. prolonged life (2-7 days)	No. survived (>7 days)
5.0	10^{-4}	2			2
	10^{-5}	4			4
	10^{-6}	7		3	4
	10^{-7}	3			3
Total		16		3 (19%)	13 (81%)
Controls	10^{-7}	10	10 (100%)		

TABLE VI.
Oral Administration of Fractionated n-Butyl Esters of Penicillin in Single Dosage.

Total amt of ester (in mg)	Dilution of culture (Strain C203Mv)	No. of mice	No. died (<48 hr)	No. prolonged life (2-7 days)	No. survived (>7 days)
4.0	10^{-4}	9	3	1	5
	10^{-5}	5			5
	10^{-6}	5			5
Total		19	3 (15.9%)	1 (5.2%)	15 (78.9%)
2.0-3.0	10^{-4}	10	2	3	5
	10^{-5}	10	3		7
	10^{-6}	10	3	3	4
Total		30	8 (26.7%)	6 (20%)	16 (53.3%)
1.0	10^{-4}	5	5		
	10^{-5}	5	2	2	1
	10^{-6}	5	2		3
Total		15	9 (60%)	2 (13.3%)	4 (26.7%)
Controls	10^{-6}	10	10		
	10^{-7}	10	10		
Total		20	20 (100%)		

which fractionated n-butyl ester was used. A single dose of varying amounts of ester was given by mouth one-half hour after infection.

As shown in Table VI, 15 of 19 mice were protected against 10^{-4}, 10^{-5}, and 10^{-6} dilutions of hemolytic streptococci by 4.0 mg of fractionated n-butyl ester. With 2.0 to 3.0 mg protection was less regular, whereas with 1.0 mg there was only slight protection.

The administration of esters by divided dosage does not affect the total amount necessary for protection. When a strain which kills within 48 hours (as C203Mv) is used, the entire therapeutic dose must be given within the first five to six hours after infection.

Similar experiments have been carried out with a type II strain of pneumococci (D/39). It was found that these esters are also effective against this organism.

Toxicity. Experiments were carried out in white mice to determine the toxicity of ethyl and n-butyl esters. Varying amounts of preparations of ethyl ester and n-butyl ester were injected into normal white mice by the subcutaneous and by the oral route. Both esters, when given in sufficient amount, produced in mice a narcotic effect resulting either in gradual recovery or in death after 2 to 30 hours. Different preparations of ester showed slight variations in the degree of toxicity. It was found that by either route of administration the LD_{50} of the ethyl ester of penicillin is about 6-7 mg per 18 g mouse, whereas the LD_{50} of the n-butyl ester ranges from 8 to 10 mg. When divided dosage is used over a period of 18 to 20 hours, the LD_{50} remains unchanged. Apparently there is a cumulative effect.

Rabbits, weighing 6-7 lbs each, have been injected by the subcutaneous and intravenous routes with alcoholic solutions containing amounts up to 100 mg of n-butyl ester with no untoward results.

Discussion. The use of penicillin has been seriously hampered by its rapid excretion and by the consequent necessity for frequent administration in order to maintain an adequate blood level. In a previous communication,[3] experiments were described in which a slow release of penicillin could be obtained in the body by the subcutaneous administration of penicillin suspended in oil or in the form of dry pellets. These methods, however, have

[3] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 285.

not been suitable for use in human therapy.

The preparation of esters of penicillin and the demonstration of their chemotherapeutic activity introduces a group of substances which can be changed from an inactive to an active form by hydrolysis within the animal body. The hydrolysis of these esters with the liberation of active penicillin takes place slowly in the body. A single injection is therefore adequate to give complete protection against hemolytic streptococcal infections in mice.

The ethyl and n-butyl esters of penicillin appear to be stable. In contrast to penicillin, they are not destroyed at the pH of the stomach and are highly active when administered by mouth. Absorption from the stomach is less regular, however, and the therapeutic dose is approximately ten times greater by the oral route than by the subcutaneous.

The toxicity of the ethyl and n-butyl esters of penicillin is 2 to 3 times that of penicillin. However, when the subcutaneous route of administration is used, the toxic range is far above the therapeutic range. When the oral route is used, the therapeutic dose more closely approaches the toxic level. In the case of the n-butyl ester the range is sufficiently wide, however, for satisfactory use of the substance.

Conclusions. Although inactive *in vitro*, ethyl and n-butyl esters of penicillin have been shown to be highly effective chemotherapeutic agents in mice infected with large doses of virulent hemolytic streptococci. These esters are apparently hydrolyzed slowly with the liberation of active penicillin. They are effective when given by the subcutaneous or by the oral route of administration.

14205

Antigenically Different Strains of Virus from a Localized Influenza Outbreak.

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The purpose of this paper is to present additional evidence that antigenically different, although related, strains of virus may be prevalent during the same outbreak of influenza. It previously has been observed that cases of influenza that occur during the same large epidemic are not all caused by antigenically identical agents.[1] The present data confirm that previous observation, and are of especial interest because they deal with an outbreak of influenza that was a particularly well localized one.

The influenza outbreak with which the investigation was concerned and which consisted of 40 clinically diagnosed cases of epidemic influenza, was observed in the nurses' infirmary of The New York Hospital during January and February, 1941. Throat washings obtained from 11 of the persons were tested for virus, and in 5 instances strains of influenza virus were isolated and adapted to Swiss mice. The 5 strains were compared with each other and with the PR8[2] by means of mouse protection tests in which each strain of virus was tested against the same group of antiserums. The antiserums were obtained from 6 rabbits each of which had been inoculated with a different strain of virus: bleedings were obtained from each rabbit 9 or 10 days and 3 to 4 weeks after a single intraperitoneal injection of a suspension containing 2% infected mouse lung in 0.85% saline. The virus suspensions used in the different protection tests were all comparable in that they contained the same magnitude of lethal doses of virus (from 300 to 600) for mice. The results of the tests are presented in Chart 1.

[1] Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

[2] Francis, T., Jr., *Science*, 1934, **80**, 457.

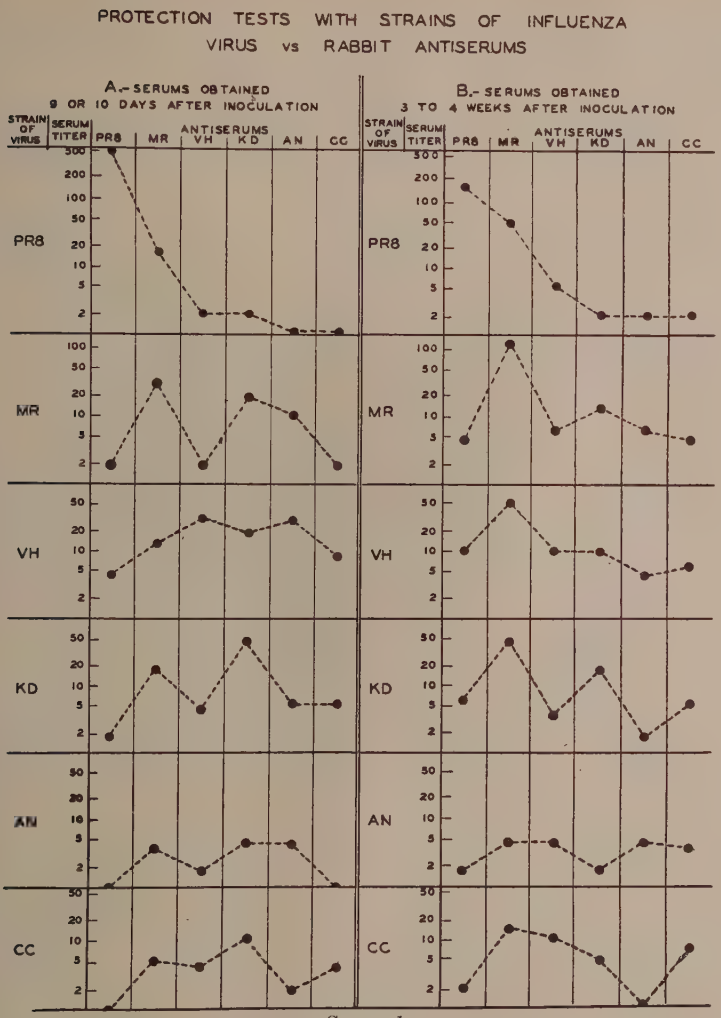

CHART 1.

In Chart 1, the results are summarized in the form of graphs in which the points represent the protective antibody titers of the different serums against the particular strain of virus. The titers are expressed as the initial dilution of serum that would protect 50% of the mice from death.[3] On the basis of the shapes of the graphs it is apparent that all of the 1941 strains (MR, VH, KD, AN, and CC) differ not only from the PR8 but also from each other. For example, in the tests against the 9- or 10-day antiserums, the MR, VH, and KD resemble each other more than they do the PR8, but in each instance the peak of the graph is at a different point; that is, each of those strains was more effectively "neutralized" by the homologous antiserum. The AN and CC strains were poor antigens and produced antiserums that had low protective capacities against even the homologous strain of virus.

As might be expected, the serums obtained 9 or 10 days after inoculation were more

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

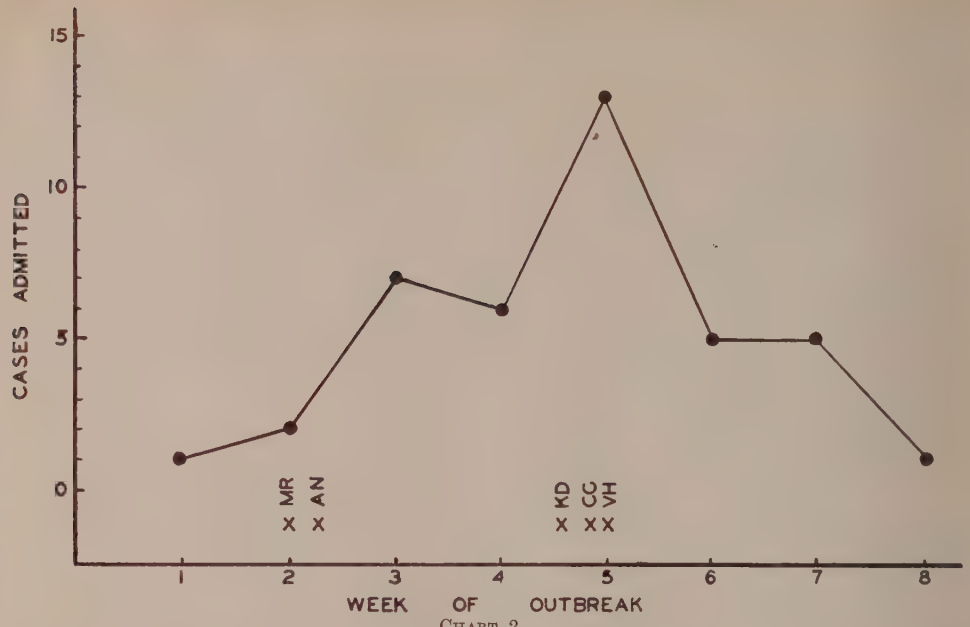

CHART 2.

specific and hence better suited to demonstrate the antigenic differences between the strains than were the serums obtained 3 to 4 weeks after inoculation. In the tests against the latter group of serums the graphs of the MR and VH strains were almost identical. However, the differences between the other 1941 strains were still evident. Furthermore, rather marked differences were still apparent between the 1941 strains as a group, and the PR8.

In view of the nature of the data presented, the relative time during the outbreak at which each of the different strains was isolated seems to be a point of interest. Chart 2 shows the total number of cases admitted to the nurses' infirmary of The New York Hospital during each week of the influenza outbreak, and also the time at which the throat washings were obtained from each person from whom a strain of virus was isolated. The antigenic characteristics seem to be unrelated to the relative time of the outbreak at which the strain was isolated. For example, strains VH and CC which were isolated from persons that were admitted to the infirmary within 24 hours of each other, seem to differ from each other as much as do any other 2 strains; whereas strains MR and VH which were isolated from persons who were ill 3 weeks apart, resemble each other rather closely.

The evidence at present available is insufficient to indicate whether a number of different but stable strains of influenza virus exist, or whether the influenza virus is a relatively unstable agent that may be altered in antigenic characteristics by the peculiar immunologic state of the hosts which it happens to infect. Regardless, however, of the reason for the existence of antigenically different strains, the present data are of interest because they show that even a well localized outbreak of influenza may consist of individual infections some of which are caused by one, some by another of a number of different although antigenically related, strains of influenza virus.

14206

Adrenal Volume in Male Rats with Reduced Glucose Tolerance.

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Cole and Harned[1] observed in a colony of the Yale strain of rats a low and erratic glucose tolerance. Subsequently, these authors[2–5] compared rats of the Yale and Wistar strains grown on the same diet and under the same conditions for 3 or more generations and reported that animals of the Yale strain exhibited a higher fasting blood sugar, a lower glucose tolerance, a greater hyperglycemic response to epinephrine, an initial resistance to insulin, a more rapid rate of growth and larger size, a higher percentage of body fat, a more irregular oestrus cycle and a higher incidence of sterility. Hyperfunction of the anterior pituitary was suggested as a common denominator for these differences.[2] Geiss[6] examined this hypothesis by the differential cell count method and found no significant difference between the anterior lobe of the pituitary glands of mature males from the two colonies. Cole and Harned had found the most pronounced differences between the two strains in the mature males. In 1942 these authors[7] compared the weights of the pituitary and adrenal glands of the two strains in relation to body weight. They found that in males of the same weight the adrenals of the Yale strain were significantly heavier than those of the controls. On the basis of this finding it appeared important to determine the volume of the adrenals of mature males of the Yale strain with the primary purpose of comparing the volume of the cortex and of the medulla with that of the controls.

Material and Methods. The adrenal glands of 10 mature male rats of the Yale strain and of an equal number of mature males of the Wistar strain matched in weight were used in the study. The average weight of the Yale rats was 345.9 g; of the Wistar rats, 349.2 g. The glands were not weighed. They were fixed in Allen's fluid. Both glands of each animal were embedded in the same paraffin block. Serial sections were cut at 10 microns; all sections were mounted and stained with hematoxylin and eosin. Both glands of each animal were studied. No effort was made to distinguish the left from the right gland.

The method of Dornfeld *et al.*[8] for the determination of the volume of the ovary of the rat was followed with minor modifications. Every fifth section of each gland was projected at a magnification of 18 and the outline of the gland and of the medulla was traced as directly projected. When necessary the cortico-medullary demarcation line was checked under the microscope. This procedure was useful occasionally in sections where the medulla was small. The area of the gland and of the medulla in the traced outline of each projected section was measured with a planimeter fixed to read in inches. The square areas of all the projection-tracings of each gland and its medulla were plotted. The square areas were used as the vertical units and the serial numbers of the sections as the horizontal. Planimetric measurement of the area under each curve was made. The volume of each gland and of its medulla was calculated from the square areas under the curves by the

1 Cole, V. V., and Harned, B. K., *Endocrinology*, 1938, **23**, 318.

2 Harned, B. K., and Cole, V. V., *Endocrinology*, 1939, **25**, 689.

3 Cole, V. V., and Harned, B. K., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 738.

4 Harned, B. K., and Cole, V. V., *Science*, 1940, **92**, 361.

5 Cole, V. V., Harned, B. K., and Keeler, C. E., *Endocrinology*, 1941, **28**, 25.

6 Geiss, M. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 121.

7 Cole, V. B., and Harned, B. K., *Endocrinology*, 1942, **30**, 146.

8 Dornfeld, E. J., Slater, D. W., and Scheffe, H., *Anat. Rec.*, 1942, **82**, 255.

TABLE I.
Volume of Adrenals—Whole Gland, Medulla, Cortex.

Yale rats (males)				Controls (males)			
	Volume in cmm				Volume in cmm		
Body wt	Adrenal	Medulla	Cortex	Body wt	Adrenal	Medulla	Cortex
226	43.9	2.6	41.3	229	18.2	1.1	17.1
	58.4	3.3	55.1		22.0	1.5	20.5
227	35.3	1.9	33.4	237	19.0	1.2	17.8
	44.9	2.8	42.1		25.5	1.4	24.1
233	26.8	1.5	25.3	256	39.7	3.2	36.5
	33.0	2.0	31.0		34.5	2.7	31.8
315	41.0	2.6	38.4	307	34.6	4.2	30.4
	50.7	3.3	47.4		31.5	3.0	28.5
383	40.5	3.6	36.9	390	20.4	1.2	19.2
	37.0	2.4	34.6		34.5	3.7	30.8
410	41.2	3.7	37.5	397	31.9	2.9	29.0
	33.9	3.1	30.8		28.9	2.0	26.9
414	24.3	1.6	22.7	400	32.4	3.7	28.7
	23.4	1.0	22.4		39.9	4.2	35.7
410	26.9	3.2	23.7	406	25.5	1.7	23.8
	29.2	2.5	26.7		31.6	2.7	28.9
415	40.4	3.2	37.2	424	13.8	1.8	12.0
	43.6	3.9	39.7		16.0	1.8	14.2
436	37.6	2.7	34.9	446	29.9	4.9	25.0
	36.6	3.1	33.5		26.5	3.1	23.4

TABLE II.
Statistical Analysis.

Adrenal	Min.	Max.	Median	Mean	Stand. dev.	Coeff. of variation	Standard error
			A. Yale Rats.				
Gland	23.4	58.4	37.3	37.4	8.6	23.0	1.9
Medulla	1.0	3.9	2.7	2.7	0.8	29.6	0.2
Cortex	22.4	55.1	34.7	34.7	8.0	23.0	1.8
			B. Controls.				
Gland	13.8	39.9	29.4	27.8	7.4	26.6	1.7
Medulla	1.1	4.9	2.7	2.6	1.1	42.3	0.2
Cortex	12.0	36.5	25.9	25.2	6.6	26.1	1.5

formula used by Dornfeld. Having computed the volume of the gland and of the medulla the volume of the cortex was taken as the difference.

Conclusions. 1. The volume of the adrenal of mature male rats of the Yale strain (rats with a reduced glucose tolerance) is significantly greater than that of the controls. 2. There is no significant difference in the volume of the medulla of the two strains. 3. The larger volume of the adrenal of the Yale rats is therefore due to the larger volume of the cortex.

14207

In vitro Action of Urea-Sulfonamide Mixtures.

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Mixtures of urea with sulfonamides have recently been employed for topical application to infected wounds.[1] Advantages of urea are that it increases the solubility of sulfonamides, and that it has strong peptizing, lytic, and deodorant actions.[2] In addition, the value of urea would be greatly enhanced if it could be shown to actually potentiate the bacteriostatic action of the sulfonamides, or to antagonize the sulfonamide inhibitors present in pus and certain body fluids. The recent experiments of Tsuchiya and colleagues indicate that the antisulfonamide action of methionine and of *p*-aminobenzoic acid can be overcome by the addition of urea to sulfonamide solutions,[3,4] and further that urea-sulfonamide mixtures are effective against sulfonamide-resistant staphylococci.[5]

The present paper is a report of experiments performed in an attempt to confirm and extend the results of Tsuchiya *et al.*, using different technical methods.

Method. The quantitative methods evolved in this laboratory in connection with studies of sulfonamide resistance have been described elsewhere in detail.[6] Briefly, growth curves were measured turbidimetrically under rigidly controlled conditions for a period of 24 hours or more. In the present studies the organism was *E. coli*, and the synthetic medium was the same as that previously described. The addition of solutions of urea, sulfathiazole, and *p*-aminobenzoic acid did not alter the pH of the basal medium.

Three types of experiments were performed, as follows:

(1) The effect of various concentrations of urea upon the growth of the organisms was determined.

(2) Various concentrations of urea were added to *p*-aminobenzoic acid-sulfonamide mixtures to determine the effect of urea upon the antisulfonamide action of *p*-aminobenzoic acid.

(3) Various concentrations of urea were added to basal medium plus sulfathiazole plus a sulfonamide-resistant strain of *E. coli* to see if urea would potentiate the action of the sulfonamide against the resistant organisms.

Results. The effect of concentrations of urea ranging from 0.20% to 2.0% upon the growth of the test organisms is shown in Fig. 1. During the phase of active growth there was definite bacteriostasis by all the concentrations of urea employed, even as little as 0.20%, and the degree of bacteriostasis was roughly proportional to the amount of urea present. When maximal growth was reached in the control tube, growth of the partially inhibited organisms continued until the same maximal level was reached.

With the sulfathiazole-*p*-aminobenzoic acid mixtures, the addition of concentrations of urea which were not bacteriostatic (less than 0.20%) did not affect the growth of the organisms. Bacteriostatic concentrations of urea inhibited growth in the sulfathiazole-*p*-aminobenzoic acid mixtures to the same extent as they did in the control tubes, but no more; the effect was purely additive. This is illustrated in Fig. 2.

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1 Holder, H. G., and MacKay, E. M., *Military Surg.*, 1942, **90**, 509.

2 Olson, M., Slider, E., Clark, W. G., and MacDonald, R., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 396.

3 Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

4 Tenenberg, D. J., Tsuchiya, H. M., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 247.

5 Tsuchiya, H. M., Tenenberg, D. J., Strakosch, E. A., and Clark, W. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 245.

6 Kirby, W. M. M., and Rantz, L. A., *J. Exp. Med.*, 1943, **77**, 29.

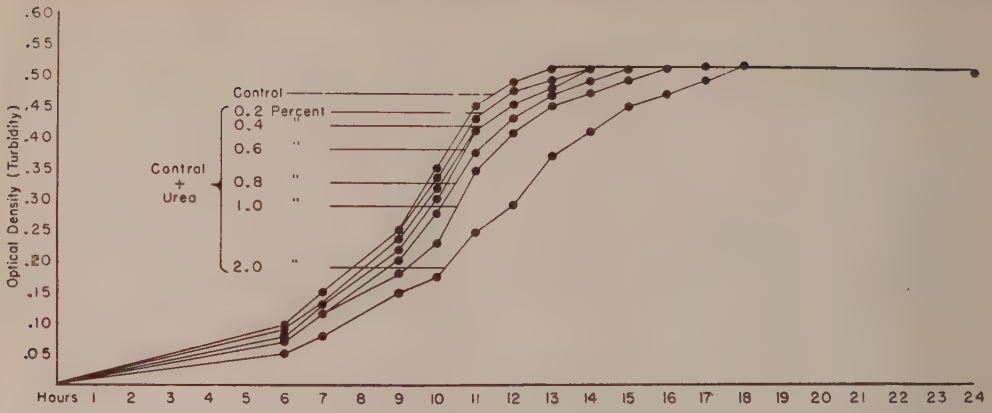

FIG. 1.
Effect of various concentrations of urea upon the growth of *E. coli*. See text for explanation.

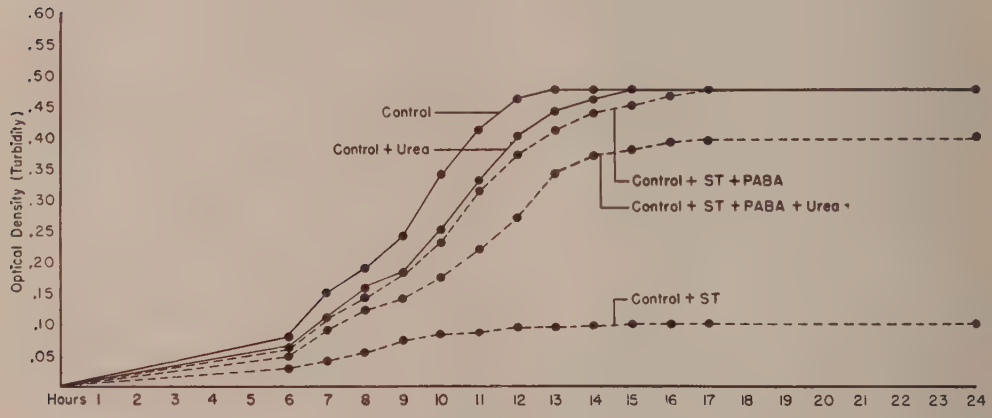

FIG. 2.
Effect upon a non-resistant strain of *E. coli* of adding urea 1% to a mixture of *p*-aminobenzoic acid (PABA) 1 mg% and sulfathiazole (ST) 10 mg%. See text for explanation.

Growth of the sulfonamide resistant organisms plus sulfathiazole was not inhibited by concentrations of urea which were not bacteriostatic. The degree of inhibition of growth produced by bacteriostatic concentrations of urea plus sulfathiazole was identical with that observed in the control tubes; here again the effect was purely additive.

Discussion. The observation of Tsuchiya and co-workers that concentrations of urea which are not bacteriostatic have an anti-sulfonamide inhibitor action has not been confirmed. The present experiments indicate that high concentrations of urea are in themselves bacteriostatic, but that concentrations of urea which are not bacteriostatic do not counteract sulfonamide inhibitors, nor do they potentiate the bacteriostatic action of the sulfonamides against resistant organisms. The conclusions of Tsuchiya's group were based upon plate counts made only at the end of 24 hours, and inspection of Figs. 2 and 3 will show why readings made at this time are not valid. Due to the limiting effect of the medium itself the partially inhibited organisms gradually catch up with the controls following the phase of logarithmic growth, so that at the end of 24 hours the bacteriostatic action of urea alone is no longer apparent, while the combined inhibition of urea plus sulfathiazole is great enough to prevent maximal growth in this tube. There is no actual potentiation of sulfonamide action;

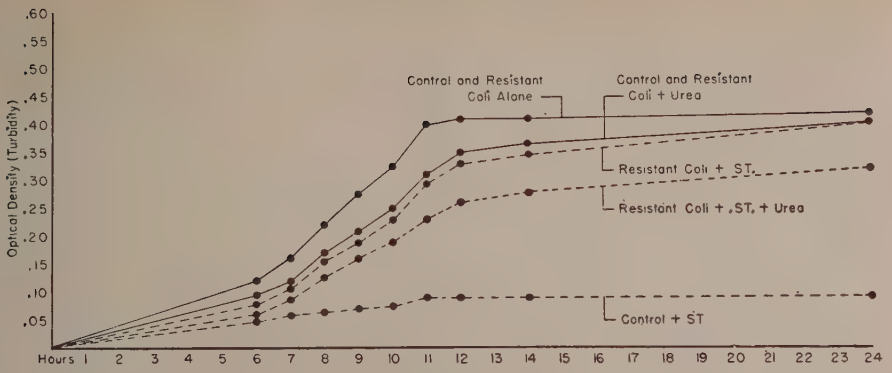

FIG. 3.

Effect upon a non-resistant and a sulfonamide-resistant strain of *E. coli* of adding urea 1% in the presence of sulfathiazole (ST) 10 mg%. See text for explanation.

the effect of urea, a bacteriostatic agent itself in high concentrations, is purely additive.

It is not to be inferred that urea-sulfonamide mixtures are of no value. On the contrary, the fact that urea is in itself a bacteriostatic agent, in addition to its solvent, peptizing, lytic, and deodorant actions, would seem to make the combination highly desirable for topical application.

Summary. Contrary to previous reports the present experiments indicate that concentrations of urea which are not bacteriostatic do not counteract sulfonamide inhibitors, nor do they potentiate the bacteriostatic action of the sulfonamides against resistant organisms. High concentrations of urea have a bacteriostatic action of their own which may be of value when they are used in combination with the sulfonamides.

14208

Presence of Hippuric Acid in Milk.

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The non-protein nitrogen, or residual nitrogen of cow's milk is known to consist in part of urea, uric acid, creatinine, amino acids, and purine bases. The remainder is composed to a large extent of compounds of unknown structure. We wish to report that one of these heretofore unidentified substances of the residual nitrogen fraction of cow's milk is hippuric acid.

In connection with the isolation of crystalline biotin from milk in this laboratory,[1] we had occasion to fractionate a biotin concentrate from milk, generously supplied by the S.M.A. Corporation of Chagrin Falls, Ohio. This material had been prepared by charcoal adsorption from a commercial milk residue which remained after the removal of protein and sugar from milk. One gram of the biotin concentrate represented approximately 15 kg of milk.

The first steps in the fractionation of this concentrate for the isolation of biotin included esterification of the concentrate with alcoholic HCl and then extraction with ethyl acetate of a slightly alkaline solution of the esterified material. The ethyl acetate extracts were concentrated to remove solvent and the residue

[1] Melville, D. B., Hofmann, K., Hague, E., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **142**, 615.

TABLE I.

	M.P.	Neutral equivalent	% nitrogen	M.P. methyl ester	M.P. *p*-bromophenacyl ester
Acid isolated from milk	188°	185	7.7	84°	151-2°
Hippuric acid	188°	179	7.8	85°	152°

was dissolved in chloroform and chromatographed on a column of Decalso.[1]

It was found that the chloroform which passed through the columns contained appreciable amounts of a substance which separated in crystalline form when the solutions were concentrated. Recrystallization of this material from an alcohol-ether mixture yielded colorless needles of a nitrogen-containing compound, m.p. 84°, which by alkaline hydrolysis yielded an acid, m.p. 188°. This acid contained 7.7% N and possessed a neutral equivalent of 185. Additional amounts of the free acid were obtained by acidification of the ethyl acetate-extracted esterification residues. These data indicated that the substance isolated from the esterified milk concentrate was the methyl ester of hippuric acid. A mixture of the isolated free acid with hippuric acid showed no depression of the melting point. Further confirmation of the identity of the isolated compound was obtained by preparation of the *p*-bromophenacyl ester from the sodium salt of the acid. The melting point of this derivative agreed with that of *p*-bromophenacyl hippurate. The data are summarized in Table I.

Quantitatively, only a minimum value for the amount of hippuric acid in milk can be derived from this work, inasmuch as there was no way of knowing how much hippuric acid had been lost during the various procedures used for the preparation of the biotin concentrate from milk, and no attempt was made to obtain a quantitative separation of the hippuric acid present in the biotin concentrate. From 75 g of concentrate, representing approximately 1160 kg of milk, there were obtained 11 g of hippuric acid; this would indicate an original minimum concentration of 10 γ hippuric acid per gram of milk.

14209

Distribution of Poliomyelitis Virus in Central Nervous System of Mice Paralyzed After Intracerebral Inoculation.*

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Tests using mice inoculated with the Lansing strain of poliomyelitis virus have been handicapped by irregularities both in morbidity and in the length of incubation periods. Some of the factors related to these difficulties such as the strain of mice[1] and the pH of the inoculum[2] have been reported. Also it has been noted that serial passage of cord and brain stem material from the first mice of an inoculated group to become paralyzed resulted in shorter incubation periods while passage from the last mice to become paralyzed resulted in longer incubation periods. The age of mice was not found to be an important

* Sponsored by the National Foundation for Infantile Paralysis, Inc.

† Fellow of the Pan-American Sanitary Bureau.

1 Smith, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 86.

2 Hammon, W. M., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

TABLE I.
Incubation Periods in Mice After Intracerebral Inoculation with Cord Suspensions in Saline of Different pH.

Intervals between inoculation and onset of paralysis	Inoculated suspension pH 7		Inoculated suspension pH 3.7
	No. of mice	% of total	No. and % of total
1-5 days	90	46	51
6-10	59	30	33
11-15	17	9	12
16-20	4	2	3
21-30	3	2	0
Survived >30	22	11	1
Total	195		100

factor in susceptibility.[3] Some workers[4] have obtained consistent results by using large pools of spinal cords from young mice which developed paralysis during the first few days after inoculation. These observations suggested that the potency of virus present in the central nervous system varied inversely with the length of incubation period. The present study was made in order to determine the amount of virus contained in the brain and spinal cord of mice which had developed paralysis at different intervals after intracerebral inoculation of the virus.

Carworth Swiss mice 3 to 5 weeks old were injected with mouse-passage virus of the Lansing strain of poliomyelitis. Each received intracerebrally 0.03 ml of a 10% suspension of infected mouse spinal cords in saline of pH 7. The numbers of mice found paralyzed or dead after inoculation during a period of observation of one month are shown in Table I. The results obtained with a smaller group of mice similarly treated, except that the inoculum was in buffered saline of pH 3.7 instead of pH 7, also are listed. It is seen that 75% of the mice which received the inoculum in saline of pH 7 developed paralysis within 10 days while 10% survived for at least one month. The respective figures for the group which was given the inoculum in saline of pH 3.7 were 85% and 1%. This effect of pH of the inoculum is in accord with results of work previously cited.[2]

The mice which were inoculated with the suspension of pH 7 and which developed paralysis after injection, were sacrificed by

TABLE II.
Virus Titer of Pooled Brains or Cords of Mice Developing Paralysis and Killed at Various Intervals After Intracerebral Inoculation.

Intervals between inoculation and onset of paralysis	Pooled material tested	No. of mice paralyzed after inoculation of 6 mice with each dilution.* Dilutions				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1-5 days	Brains	6	4	3	0	0
	Cords	5	6	5	2	0
6-10	Brains	2†	0	0	0	0
	Cords	6	5	4	2†	0
11-20	Brains	0	0	0	0	0
	Cords	6	5	4	2	0
21-30	Brains	2†	0	0	0	0
	Cords	6	5	4	2	0

* Observed for 60 days.
† Found dead.

[3] Young, L. E., and Merrell, M., *Am. J. Hyg.*, 1943, **37**, 80.

[4] Kramer, S. D., Personal communication.

etherization. First the brain, then the spinal cord, the latter including the medulla, were removed separately and stored whole in dry ice until used. Incidental results of titrations of infected mouse spinal cords after various conditions of storage indicated that virus might remain at its original titer for at least 3 weeks when kept as a 10% suspension in saline at 4° or −70°C.

Material from the mice killed during each successive interval of 5 or 10 days was pooled. These pools of brains and spinal cords, respectively, were tested at one time by intracerebral injection of groups of 6 mice with serial dilutions in saline of pH 7. The results are shown in Table II. It is evident that mice which became paralyzed during the first 5 days after inoculation had essentially the same amount of virus in the brain as in the spinal cord-medulla. After 5 days, however, little or no virus was recovered from the brain although the various pools of cord and medulla collected at different intervals up to 30 days contained essentially the same amount of virus at all times.

Summary. Mice which developed paralysis within 5 days after an intracerebral inoculation of Lansing strain of poliomyelitis virus had approximately as much virus in the brain as in the spinal cord-medulla. After 5 days little or no virus was recovered from the brain although pools of cord-medulla of mice which became paralyzed up to 4 weeks after injection were found to contain as much virus as did the pool of the first 5 days.

14210

Delayed Lethal Effect of X-rays on Fibroblasts Cultivated *in vitro*.

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Studies on the effect of X-rays upon cells growing *in vitro* have shown that with sufficiently high X-ray doses the outgrowth of the irradiated cell culture can be checked immediately and completely. For colonies of chicken fibroblasts, 250,000 r units are the minimum dose which produces this effect ("the immediate lethal dose").[1]

It is also possible to produce a lethal effect on cells *in vitro* by exposure to considerably smaller X-ray quantities. X-ray doses, which are themselves too low to cause immediate cessation of growth of the irradiated culture, are capable of exerting a lethal action after a latent period (Fischer and Baastrup,[2] Santesson,[3] Spear,[4] Cox[5]). Cultures treated with such X-ray doses continue to grow for some time; but after a certain number of passages, cell growth ceases.

The mechanisms of action of the immediate and the delayed lethal doses, respectively, are, in all probability, fundamentally different. The immediate lethal doses stop the outgrowth in the irradiated culture by altering the motility of the cells. The delayed lethal doses act, on the other hand, by affecting a more susceptible cell function, namely, cell division. Cultures treated with these doses die in the course of subsequent transfers because the cells become incapable of multiplication.

In a previous communication[6] we reported on the delayed effect on cell cultures of X-ray

[1] Doljanski, L., and Goldhaber, G., *Growth*, 1942, **6**, 235.

[2] Fischer, A., *Gewebezüchtung*, München, Müller & Steinicke, 1930.

[3] Santesson, L., *Upsala Läkareförenings Förhandlingar*, 1928, **34**, 591.

[4] Spear, F. G., *Proc. Roy. Soc., B* (London), 1930, **106**, 44.

[5] Cox, S. F., *Brit. J. Radiol.*, 1931, **4**, 111. 1942, **6**.

[6] Goldhaber, G., and Doljanski, L., *Growth*, 1942, **6**.

doses of 2,500, 5,000, 12,500, 25,000, and 50,000 r units. It was shown that treatment with 2,500 r units causes a depression in the growth rate of the irradiated culture. This depression is transient and is gradually overcome in the course of subsequent passages. The effect of treatment of cell cultures with doses of from 5,000 r units to 50,000 r units is entirely different. Doses of this magnitude inhibit cell proliferation irreversibly. Cultures treated with these doses, although they develop well after irradiation and differ little from cultures irradiated with 2,500 r units in both size and appearance, are destined to die. They can be cultivated for some time, but succumb after a number of passages. Cultures irradiated with doses between 5,000 to 25,000 r units have an average survival of 5.5 passages; cultures irradiated with 50,000 r units survive 3.1 passages.

In view of the particular biological and clinical significance of irradiations causing a delayed lethal effect on cells, an attempt was made to determine, exactly, the minimal X-ray dose exerting this effect.

Material and Methods. (a) *Culture technic.* Our experiments were carried out on standardized cultures of chicken fibroblasts derived from heart of 7-day-old embryos. The cell colonies were cultivated in hanging drops according to the usual procedure, using a mixture of fowl plasma and embryo extract as the medium. Growth curves of the cultures were constructed by the planimetric measurements of outline drawings of the surface area made every 24 hours.

(b) *Radiological technic.* Irradiations were carried out using a demountable X-ray tube which worked at a tension of 35 KV on currents of 2 MA. The tube had a copper-anticathode and a window of aluminum foil, 30 μ in thickness. Absorption analysis showed that the rays penetrating through the window foil and the 0.03 mm thick mica-coverglass of the cultures were mainly copper K-rays. The X-ray intensity at the distance of the irradiated subject was about 8,500 r/min for a tube current of 2 MA.

(c) *Experimental procedure.* Fibroblast cultures were irradiated, immediately after transfer, with the following X-ray doses: 3,000, 4,000, and 5,000 r units. The irradiated cell colonies were cultivated throughout successive passages. Sub-cultures were made at intervals of 3 days, and this routine was continued as long as the cultures survived, or until full recovery had been attained.

Results. The results of the experiments

TABLE I.

Irradiation with 3,000 r units		Irradiation with 4,000 r units		Irradiation with 5,000 r units	
Exp. No.	Results	Exp. No.	Results	Exp. No.	Results
23199	+	23198	—(6)	19893	—(7)
23201	—(4)	23200	—(4)	19894	—(6)
23782	+	23202	—(4)	20141	—(7)
23788	—(2)	23203	+	20142	—(7)
23789	+	23785	—(5)	20143	—(7)
23790	+	23786	—(5)	20300	—(5)
23998	+	23787	—(5)	20662	—(3)
23999	+	23793	—(7)	20808	—(3)
24000	+	24003	+	20809	—(4)
24001	+	24004	+	20810	—(5)
24002	+	24005	+	20811	—(4)
		24006	—(5)	20812	—(5)
		24480	+	24481	—(5)
		24482	+	24483	—(5)
		24486	—(5)	24485	—(5)
		24488	—(5)	24487	—(6)
		24490	—(6)	24489	—(3)
				24491	—(6)

\+ Prolonged cultivation possible.
— Prolonged cultivation impossible.
Bracketed figures represent the number of passages through which cultivation of the irradiated cell culture could be carried.

(Table I) may be summarized as follows: Irradiation with 3,000 r units does not produce permanent damage of the cell culture; with few exceptions, cultures exposed to this dose survive and after several passages show a quite normal rate of growth. Irradiation with 4,000 r units produces variable results; out of 17 cultures treated with 4,000 r units, 11 cultures succumbed in the course of the succeeding transfers, while 6 cultures survived. Irradiation with 5,000 r units renders prolonged cultivation of the cell colonies impossible. A dose of 5,000 r units is, therefore, to be regarded as the minimal "delayed lethal dose" for chicken fibroblasts.

14211

Effect of Biotin Deficiency on Duration of Infection with *Trypanosoma lewisi* in the Rat.*

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Before August, 1942, when this study was undertaken, there had been few reports on the effect of a vitamin deficiency on the course of a parasitic protozoan infection. Since much work on biotin deficiency had already been done in this laboratory this vitamin was thought suitable for the purpose. It was planned, therefore, to determine the effect of biotin deficiency on the course of infection with a blood protozoan.

Trypanosoma lewisi was chosen because it was considered advisable to use an organism whose course of infection had already been well studied.[1]

The rats used in these experiments were white albinos bred for the most part in this laboratory. The experimental rats were kept on a modified egg white diet consisting of:

	g
Cornstarch	185
Salt mixture	50
Peanut oil	150
Cod liver oil	30
Water	25
Egg albumen†	300
Sugar	185

They were also given the following vitamin supplement daily: thiamine, 20 γ; pyridoxine, 20 γ; riboflavin, 25 γ; calcium pantothenate, 100 γ. In addition the experimental rats were given 50 mg of choline chloride thrice weekly. The food intake was high in all cases until the terminal phases of the biotin deficiency. The control rats were kept on a modified Sherman diet,[2] with liver or beef twice a week and cabbage once a week in addition.

All rats in a specific group were inoculated intraperitoneally with the same number of *Trypanosoma lewisi.*‡ However, the numbers of parasites in these inocula were only roughly approximate from experiment to experiment. Examination of the blood, tail blood or if necessary, heart blood, was carried out every other day until the end of the infection. After the first disappearance of the parasites from the blood the rats were followed for 6 days and then observations were discontinued. It should be noted that these rats were not *Bartonella* free, but since this was true of both the experimentals and the controls it was felt that the differences in the 2 groups can be safely ascribed to the differences in their diet.

* Under a grant from the Rockefeller Foundation.

1 Taliaferro, W. H., "The Immunology of Parasitic Protozoa" in *Protozoa in Biological Research*, edited by G. N. Calkins and F. M. Summers, Columbia University Press, 1941.

† Purchased from the Weideman Co., Cleveland, Ohio.

2 Smith, A. H., and Bing, F. C., *J. Nutrition*, 1928, **1**.

‡ Our strain of *Trypanosoma lewisi* was supplied us through the courtesy of Dr. W. H. Taliaferro of the University of Chicago.

Experimental Studies. Group I consisted of animals in an advanced stage of biotin deficiency used in the preliminary experiments. These animals either had survived to the end of their infection or to 30 days after inoculation. The controls were inoculated over the same period of time and had been intended for controls in the groups which were subsequently amalgamated. Due to circumstances beyond our control at this time the normal rats were not selected as to age and weight to be the equivalents of the experimental rats. The 10 experimental rats had a mean length of infection of 34.9 $\pm$ 2.97 days based on the following individual lengths of infection: 14, 30, 30, 33, 35, 37, 38, 40, 43, 49 days. The 13 control rats had infections of 7, 8, 9, 9, 10, 13, 13, 21, 24, 24, 26, 28, 28 days respectively which gave a mean length of infection of 16.9 $\pm$ 2.3 days. The value of t for this group was 4.874 $>$ 2,831 for 21°'s of freedom, P = .01§

The rats with the biotin deficiency thus showed a significantly longer course of infection than the normal rats. However, due to the inadequacy of the controls a similar series was repeated.

In Experiment II 9 rats with moderate to advanced symptoms of biotin deficiency were used.‖ Four of these died before the 20th day and are not used in the data.¶ Five rats of the same age which had been kept on the Sherman diet were used as controls.

The 5 biotin deficient rats had infections of 35, 39, 39, 39, 41 days in length which resulted in a mean length of 38.6 $\pm$ 1 day. The 5 control rats had a mean length of infection of 21.8 $\pm$ 1.5 days based on individual infections of 19, 20, 20, 25, 25 days in duration. The value for t in this experiment was 9.38 $>$ 3.355 for 8°'s of freedom, P = .01.

These results in which the controls are adequate definitely confirm those of Group I. Rats exhibiting moderate to severe symptoms of biotin deficiency have a significantly longer course of infection than the normal rats.

Experiment III was carried out in order to determine whether the same delay in termination of the infection would be seen in rats showing only slight symptoms of biotin deficiency. Two groups of rats were used in the experiment. The first group was composed of 5 rats** from the same lot as those used in Experiment II and the controls were normal rats of the same group. The second group was composed of 7 slightly larger rats†† which showed about the same symptoms of biotin deficiency and its controls were of approximately the same age. The same amount of inoculum was given intraperitoneally to both groups and their controls.

The lengths of infections in the 8 surviving experimental rats were 31, 34, 36, 36, 37, 37, 37, 41 days respectively which resulted in a mean length of infection of 36.12 $\pm$1. The 4 controls had a mean length of infection of 22.5 $\pm$ .5 days based on infections of 22, 22, 22, 24 days in duration. The value of t for Experiment III was 9.06 $>$ 3.169 for 10°'s of freedom, P = .01. Therefore, the rats showing slight degrees of biotin deficiency had as great a delay in terminating their infections, compared to the normal rats, as did the rats showing moderately severe symptoms.

Experiment IV was devised to see whether rats having latent biotin deficiency would also have a prolonged infection. A litter of 9 rats was divided into 2 groups. The first group of 6 rats was fed the egg-white diet. The remaining 3 were put on the Sherman diet. They were infected as soon as the majority of the experimental rats were beginning to show initial symptoms of biotin deficiency.

In this experiment the 6 experimental rats had infections of 25, 27, 27, 31, 37, 45 days in length which resulted in a mean length of infection of 32 $\pm$ 3.1 days. The 3 control rats had a mean length of infection of 17.3 $\pm$ 2.6 days based on individual infections of

§ The authors are indebted for aid in statistical analysis to Dr. Norman C. Wetzel, Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University.

‖ One had slight symptoms of biotin deficiency and subsequently improved.

¶ Six other rats with farther advanced symptoms were inoculated at the same time but all of them died before the 20th day.

** Three died.

†† One died.

12, 20, 20 days respectively. The value for t in this case was $3.0002 < 3.499$ for $P = .01$. However, t was $3.000 > 2.998$ for the value of $P = .02$. Thus the difference between the controls and the experimental rats was not as significant and clear cut as in the preceding experiments. The value for $P = .02$, on the other hand, indicates there was a slightly significant prolongation of infection. This experimental group, as might have been expected, can, therefore, be said to occupy an intermediate position between normal rats and those showing definite symptoms of biotin deficiency.

A greater dispersion as to length of infection occurred in this group. The heaviest rat in the group, with a weight of 120 g at the beginning of the infection, became negative first, in 25 days; the lightest rat, with a weight of 92 g at the beginning of the infection, became negative last, in 45 days. A further point of interest was that the experimental rats showed no increase in their slight symptoms of biotin deficiency while they were infected with *T. lewisi*. Even more interesting is the fact that the experimental rats began to show signs of the deficiency in almost the exact order in which they became negative.

Experiment V was done to determine whether the prolongation of infection in the biotin-deficient rats was due specifically to biotin or to the lack of some other element in the diet. Litter-mates from 2 litters born on the same day were used in the next experiment. Five rats from one litter and 3 rats from the other were placed on the deficient diet,‡‡ while 3 rats from the first litter and 2 rats from the second were given the Sherman diet.§§ When slight to moderate symptoms of biotin deficiency appeared in all the experimental rats they were infected with *Trypanosoma lewisi*. Each deficient rat was given .5 γ of biotin subcutaneously daily, beginning at the time of inoculation and continuing until the infection was terminated. The 4 surviving deficient rats which had been treated with biotin had infections of 14, 26, 28, and 29 days in length respectively which resulted in a mean length of infection of 24.25 ± 3.46 days. Their 4 controls had a mean length of infection of 21 ± 2.6 days resulting from individual infections of 17, 17, 22, 28 days respectively. There was no significant difference between the biotin-deficient rats and their controls.

Since there was no significant difference between the 23 biotin-deficient rats of Experiments I to III, for purpose of comparison they were amalgamated and their mean length of infection, 36.13 ± 1.35 days, determined. The data from all the 29 normal rats in all 5 experiments was similarly treated and their mean length of infection 19.8 ± 1.2 determined. Since the 6 latent deficient rats occupied an intermediate position they were not included in computation of the mean of the biotin-deficient rats.

Comparing the 4 deficient rats which had been treated with biotin during their course of infection with the 29 normals from all 5 experiments, it was seen that there was no significant difference between the mean lengths of the infections 24.25 ± 3.46 and 19.8 ± 1.2. The value for t in this case is $1.25 < 2.57$ for $P = .01$ for 31°'s of freedom. A similar comparison with the latent biotin-deficient rats with a mean length of infection of 32 ± 3.1 days showed no significant difference between them and the deficient rats treated with biotin which had a mean length of 24.25 ± 3.46 days; t is $1.62 < 3.35$ for 8°'s of freedom, $P = .01$ in this case. However, the slight difference that occurs is toward the normal side. While a comparison of the 23 biotin-deficient rats, with a mean length of infection of 36.13 ± 1.35 days, with the 4 biotin-deficient rats which had been treated with biotin, having a mean length of infection of 24.25 ± 3.46 days, showed a definite and significant difference. t in this case had a value of $5.46 > 2.785$ for 25°'s of freedom, $P = .01$.

Discussion. From the above data it seems conclusive that biotin deficiency results in great prolongation of infection with *T. lewisi* in the rat. This effect does not seem dependent on the severity of the symptoms as long as the symptoms are definite. Latent biotin deficiency also results in prolongation but of less significant degree. Treatment of

‡‡ Four of which died.

§§ One of which died.

the deficient rats with biotin results in shortening of the lengths of their infections to a point where they are not significantly different from the normals.

With regard to the lack of appearance of further symptoms noted in the latent biotin-deficient rats of Experiment IV, it should be noted that a number of the moderately biotin-deficient rats able to withstand the added strain of infection with *T. lewisi,* had already given the impression of improvement of their deficiency symptoms at or shortly after the height of infection. These phenomena led to the hypothesis that the parasites might be capable of producing sufficient biotin to prevent much aggravation of the deficiency symptoms of their host. Experiments are now in progress to definitely determine whether there is such an effect.

The effect of biotin deficiency in prolonging the infection with *Trypanosoma lewisi* in the rat makes Trager's recent paper[3] indicating a similar lengthening of infection in avian malaria by means of the same deficiency more interesting. This is especially so because the same effect was noted in the Sporozoa as is here noted in Mastigophora.

Studies of the stained material are now being worked out in detail to determine the effects of biotin deficiency on the parasite numbers and on the antibody sequence. Further work is also being done to determine the effect of excess biotin on the infection in the normal rat. It should be noted that since this work was done exclusively with biotin deficiency no claims are made at present that such an effect is specific to biotin.

Summary. Biotin deficiency has been found to prolong the infection with *T. lewisi* in the rat. This effect can be negated by the administration of biotin to the deficient rat during the course of infection. Biotin appears to be instrumental directly or indirectly in the activation of the immune bodies in this infection.

[3] Trager, W., *Science,* 1943, **97**, 206.

14212 P

Production of Rh Antiserum by Inoculation of Guinea Pigs with Human Erythrocytes.

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Antisera used for detecting the presence of the Rh antigen in human erythrocytes have been of two types: the serum of guinea pigs immunized against the red blood cells of *Rhesus* monkeys,[1,2] and the serum of certain Rh— human beings who have developed antibodies against the Rh antigen.[3,4] These latter sera are not readily procurable since human beings possessing the Rh antibody are infrequently encountered and accordingly the incidence of their employment is greatly restricted. Guinea pig anti-rhesus sera also have limitations to their usefulness. Certain differences have been noted in the Rh antigen as found in monkey and human erythrocytes,[5,6] and the implications of these differences as yet have not been clarified. Further, the production of such sera in certain laboratories may be hampered by the present war-time scarcity of *Rhesus* monkeys. For these practical reasons, as well as for certain theoretical con-

[1] Landsteiner, K., and Wiener, A. S., *J. Exp. Med.,* 1941, **74**, 309.

[2] Gallagher, Fred W., and Jones, Lloyd R., *J. Immunology,* 1943, **46**, 9.

[3] Levine, P., Katzin, E. M., and Burnham, L., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 346.

[4] Wiener, A. S., and Forer, S., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 215.

[5] Davidsohn, I., and Toharsky, B., *Am. J. Clin. Path.,* 1942, **12**, 434.

[6] Fisk, Roy T., and Foord, Alvin G., *Am. J. Clin. Path.,* 1942, **12**, 545.

siderations, we have undertaken the production of Rh antisera by inoculating experimental animals with Rh+ human erythrocytes.

Guinea pigs were inoculated with varying doses of red blood cells over a period of 3 months. The cells used were from an Rh+, group O, type MN individual. The presence of the Rh antigen in these cells was assured by the fact that they agglutinated in several absorbed guinea pig anti-rhesus sera and in 6 human anti-Rh sera. Immune serum obtained in this manner when absorbed, according to our previously reported technic[2] with group O, type MN, Rh— cells, distinguished sharply between known Rh+ and Rh— human erythrocytes of all blood groups.

In view of these findings it would appear that the possibilities for producing Rh antisera, useful in testing the red blood cells of human beings belonging to any blood group, have been greatly extended.

14213

Effect of Propylene Glycol on Bacterial Spores.

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Recent publications[1,2] have demonstrated the bactericidal and viricidal activity of propylene glycol. Using air-suspended bacteria as well as broth cultures *in vitro*, the following organisms have been found susceptible to this substance: Hemolytic and non-hemolytic staphylococci, *B. coli*, hemolytic streptococci, *Streptococcus viridans*, pneumococci (Types I, II, and rough), *Hemophilus influenzæ*, *B. pertussis* and PR8 strain of influenza virus.

Bactericidal action is demonstrable in dilutions as high as one part of propylene glycol in 50 million parts of air when air-suspended organisms are tested, while *in vitro* experiments demonstrate that organisms grow in broth containing as much as 15% glycol but are killed when the concentration of glycol in the media reaches 85%.

In view of the widespread interest in the use of glycol vapor as a means of controlling cross infections, this study was carried out in an attempt to throw further light on its possible mode of bacterial killing. Using *B. subtilis* as the test organism, observations were made to determine the effect of propylene glycol on bacterial spores.

I. *Effect of Propylene Glycol Vapor on Air-Suspended Bacteria.* These tests were done in small glass chambers of 2 cubic feet capacity; the air was agitated by means of small fans and standardized broth cultures were atomized into the space. Measured dilutions of propylene glycol vapor were introduced. Samples of 2 liters of air were taken for culture and analysis.

A. *Vegetative Forms.* Eighteen-hour cultures of *B. subtilis* were sprayed into the chambers and results similar to those reported occurred; *i.e.*, there was immediate killing in dilutions as high as 1 g of glycol in 25 million cc of air.

B. *Spores.* A 72-hour culture of *B. subtilis* showing many sporulated forms was heated for 20 minutes at 80°C. After this period of heating a loop of the culture was inoculated in broth. Abundant growth occurred, showing that the spores were viable. An 18-hour culture showing no spore forms was killed by this amount of heat. When the suspension of spores was atomized into "glycolized air" no bactericidal effect was observed even when the introduction was made into a saturated

1 Robertson, O. H., Bigg, E., Puck, T. T., and Miller, B. F., *J. Exp. Med.*, 1942, **75**, 593.

2 Robertson, O. H., Loosli, C. G., Puck, T. T., Bigg, E., and Miller, B. F., *Science*, 1941, **94**, 612.

atmosphere (1 g of glycol in 200,000 cc of air).

II. *Effect of Propylene Glycol in Vitro.* A. *Vegetative Forms.* Inoculation of glycol into broth cultures resulting in glycol concentrations ranging from one to 100% produced the expected effects. Growth occurred in dilutions up to 20% and killing was obtained in solutions of 80% or more.

B. *Spores.* No effect on the viability of spores resulted from the inoculation of glycol into these cultures even though spores were exposed to the glycol for as long as 72 hours.

Discussion. Although no actual proof has been demonstrated as to how the glycols exert their bactericidal action, it would appear that it is brought about by the chief physical property of glycol, namely, hygroscopicity. Due to the moisture contained in each droplet of suspended bacteria and/or the film of moisture about each bacterial cell, molecules of glycol are attracted. This develops a high glycol concentration in direct contact with the cell membrane. As shown in test-tube experiments these high concentrations produce bacterial death. The lethal effects may take place by actions which possibly include dehydration of the cell, interference with respiration, denaturation of protein, inhibition of enzyme activity, and others. Since spores are particularly resistant to drying, the possibility is suggested that removal from, or entrance into the cell body, of water, would be difficult, whereas in vegetative forms this action could occur more readily, mediated by a high glycol concentration on the cell surface.

Conclusion. Propylene glycol in its liquid or vapor state demonstrates no killing effect on spores of *B. subtilis.* This observation tends to support the hypothesis that the bactericidal action of the glycols is due to the hydroscopic properties of these substances.

14214

Effect of Hormones on Contraction of Striated Muscle and on Choline Esterase Activity.

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While sex hormones and some of the hormones secreted by the hypophysis do not contract striated muscle in low concentrations, they may modify the biochemical equilibrium of the muscle. The purpose of this study is to investigate whether or not the presence of the above substances modifies the contraction of the muscle produced by known chemical agents.

Experimental. Effect of the Hormones on the Acetylcholine Sensitivity of Striated Muscle. The *Rectus abdominis* muscle of the frog was excised and suspended in a muscle chamber containing 20 cc of Ringer's solution at pH 7 and room temperature. The muscle was immersed alternately in a 0.1 mg per 100 cc acetylcholine solution for 30 seconds and in Ringer's solution for 10 minutes until the height of contraction remained constant for 3 cycles. This contraction served as control. The height of contraction was registered with an isotonic lever on a kymograph. The muscle was then immersed in a solution or suspension of one of the hormones in Ringer's solution for 5 minutes before each immersion in acetylcholine. The hormones were used in increasing concentrations. Changes in the height of contraction of the muscle due to the presence of the hormones were measured and expressed as percent of control. Experiments were performed in each case using instead of hormones the solvents alone. The solvents alone (sesame oil, peanut oil, and water with acacia) did not modify the effect of acetylcholine on the muscle. Other solvents (alcohol, ether, acetone, bile salts) were not used

TABLE I.
Effect of Hormones on Muscle Contraction Due to Acetylcholine.

Substance	Solvent	No. of exp.	Increase of height of contraction in % of control (mean values*) Conc. in I.U. per 100 cc Traces	1	10	100
Follutein (ant. pituitary-like sex hormone, Squibb)	water	20		33	40	52
Estrone Suspension (follicular hormone, Abbott)	water and acacia	12		10	20	27
Theelin (follicular hormone, Parke-Davis)	water	10				27
Estradiol (follicular hormone, Ciba)	,,	5	20			
Progestin (corpus luteum hormone, Roche-Organon)	peanut oil	15	18			
Progesterol (corpus luteum hormone, Ciba)	water	8	19			
Testosterone Propionate (Ciba)	,,	12	22			
Testosterone Propionate (Schering)	sesame oil	19	20			
Pitressin (vasopressor and anti-diuretic hormone, Parke-Davis)	water	18		17	15	
Pitocin (oxytocic hormone, Parke-Davis)	,,	10		—1	2	

			Conc. in mg per 100 cc 0.0001	0.001	0.01	0.05	0.1	0.5	1
Physostigmine	water	10	0	13	15	19	37	79	175

* The S.E. of the mean for each value was less than ±10%.

because they strongly potentiate the contraction producing effect of acetylcholine.[1–7]

The height of contraction of muscle due to acetylcholine was slightly potentiated by most of the hormones used (Table I). The potency of these hormones may be evaluated by comparison with physostigmine. Low concentrations are as potent as low concentrations of physostigmine. However, an accurate quantitative comparison to physostigmine is impossible because the slight water solubility of some of the hormones prevents the evaluation of the amount dissolved and the use of more concentrated solutions.

Follicular hormone, Progesterone, Follutein, Testosterone, and Pitressin potentiated the acetylcholine contraction of the *Rectus abdominis* muscle from 15 to 40% more if mixed with brain extract or urine than did brain extract or urine alone. Pitocin did not modify the contraction producing power of brain extract or urine. Experiments using the same hormones in the same solvents, but inactivated by heat or oxydation, were also performed and the results demonstrated that the effect of the hormones on the muscle contraction induced by acteylcholine was due to the hormones themselves.*

The potentiation of the effect of acetylcholine may be due to at least two processes: (1) increased sensitivity of the effector cells to stimuli, and (2) decreased inactivation of acetylcholine by tissue choline esterase. The first possibility was investigated by studying the effect of the above hormones on the response of the muscle to potassium, the second with choline esterase experiments.

Effect of the Hormones on the Potassium Sensitivity of Striated Muscle. The *Rectus abdominis* muscle was treated as described

1 Meng, *Chin. J. Physiol.*, 1940, **15**, 143.

2 Bernheim and Bernheim, *J. Pharm. Exp. Therap.*, 1936, **57**, 427.

3 Ettinger, Brown and Megill, *Ibid.*, 1941, **73**, 119.

4 Torda, *Ibid.*, 1943, **77**, 50.

5 Torda, *Ibid.*, 1943, **77**, 350.

6 Torda, *Ibid.*, in press.

7 Sobotka and Antropol, *Enzymologia*, 1937, **4**, 189.

* The results are not included because various aspects of the process of inactivation require further investigation and are not relevant to this study.

TABLE II.
Effect of Hormones on Muscle Contraction Due to Potassium.

Substance	Solvent	No. of exp.	Increase of height of contraction in % of control (mean values*) Conc. of substances in 100 cc Traces	0.01 mg	0.05 mg	1 I.U.	10 I.U.	100 I.U.
Follutein	water	20				29	45	99
Estrone Suspension	water and acacia	12				24	29	43
Theelin	water	10						40
Estradiol	"	5	38					
Progestin (Roche-Organon)	peanut oil	12	26					
Progesterol (Ciba)	water	8	52					
Testosterone Propionate (Ciba)	"	12	40					
Testosterone Propionate (Schering)	sesame oil	20	36					
Doca (desoxycorticosterone, Roche-Organon)	" "	10	46					
Cholesterol	water	20		47	60			
Pitressin	"	18				17	20	
Pitocin	"	10				2	1	

* The S.E. of the mean for each value was less than ±10%.

above but was contracted by a 20 mM potassium solution instead of acetylcholine. Most of the hormones used increased the potassium sensitivity of the *Rectus abdominis* muscle (Table II). Results paralleling the above were obtained using desoxycorticosterone or cholesterol, indicating that the potentiation of the potassium sensitivity may be a general property of the sterol compounds.

The solvents alone (sesame oil, peanut oil, and water with acacia) did not modify the effect of potassium on the muscle. Other solvents (alcohol, ether, acetone, bile salts) were not used because they strongly potentiate the contraction producing power of potassium. Follicular hormone, Progesterone, Follutein, Testosterone, and Pitressin potentiated the contraction of the *Rectus abdominis* muscle from 15 to 50% more if mixed with brain extract or urine than did brain extract or urine alone. Pitocin did not modify the contraction producing power of brain extract or urine. Experiments using the same hormones in the same solvents, but inactivated by heat or oxydation, were also performed and the results demonstrated that the effect of the hormones on the muscle contraction induced by acetylcholine was due to the hormones themselves.*

Experiments with Choline Esterase. (A) *Biological Method.* The choline esterase of steer brains was concentrated by the method of Bernheim and Bernheim.[2] Acetylcholine was added to the suspension of choline esterase to form a final concentration of 0.1 mg per 100 cc. The *Rectus abdominis* muscle was immersed for 30 seconds in this mixture immediately after the addition of acetylcholine and again 10 minutes later. The suspension of choline esterase was prepared in such concentration that 50% of the acetylcholine added was destroyed during the 10 minutes of contact. The same procedure was followed with mixtures containing hormone, choline esterase and acetylcholine.

(B) *Experiments with Warburg's Method.* Brains of steer, rabbit and pig were frozen and finely ground. The tissue was pressed through muslin and suspended in bicarbonate Ringer's solution at pH 7.4. Two cc of the suspension with or without hormone were placed in the body of the vessel and 0.2 cc of a 5 mg per 100 cc acetylcholine solution was placed in the side cup. The choline esterase activity of the brain was then determined by the method of Warburg (Ammon[8]). The solvents alone (sesame oil, peanut oil, and water with acacia) did not modify the activity of choline esterase.

The results of the biological test indicate that hormones potentiating the acetylcholine contraction of the striated muscle decrease the activity of choline esterase (Table III). These

[8] Ammon, *Pflüger's Arch.*, 1933-34, **233**, 486.

TABLE III.
Effect of Hormones on the Choline Esterase Activity. (Biological Method.)

Substance	Conc. in 100 cc	Decrease of activity of choline esterase in % of control: Mean (of 5 exp.)	S.E. of mean
Follutein	100 I.U.	—26	±2.0
Estrone Suspension	500 "	—23	±2.9
Progestin	Traces	—20	±2.3
Testosterone Propionate	"	—21	±1.9
Pitressin	50 I.U.	—12	±2.2
Pitocin	50 "	0	±1.0

TABLE IV.
Effect of Hormones on the Choline Esterase Activity. (Warburg's Method)

Substance	Conc. used	No. of exp.	Decrease of activity of choline esterase in % of control: Mean	S.E. of mean
Follutein	5 I.U.	25	—24	±1.8
Estrone Suspension	100 "	10	—22	±2.1
Progestin	1 "	15	—18	±1.6
Testosterone Propionate (Schering)	25 mg	10	—19	±1.9
Pitressin	2 I.U.	10	—13	±2.2
Pitocin	5 "	10	0	±2.2

observations were confirmed by the chemical test (Table IV). An evaluation of the anti-choline esterase potency of the above substances may be attempted by comparison with the anti-choline esterase activity of physostigmine considering that $2.5/10^4$ mg% physostigmine inhibits the choline esterase activity by 50%.[8] This evaluation is of necessity an approximate one because the very slight water solubility of most of the hormones used prevents an accurate evaluation of the amount dissolved or the amount entered in contact with the enzyme.

Discussion. Some of the sex hormones and some of the hormones secreted by the hypophysis potentiate the sensitivity of the effector cells to certain chemical stimuli and decrease the choline esterase activity of the tissue. The lowest concentrations required to exert these effects are comparable with biological concentrations of the hormones. It is possible that the potentiation of the response of the *Rectus abdominis* muscle to chemical stimuli is due to impurities of the substances used or to physicochemical properties of the large molecules of the hormones distributed in the extracellular spaces, but experiments of Pompen[9] and Reynolds and Foster[10,11] show that at least the follicular hormone has a cholinergic effect *in vivo*. The above results suggest that sex hormones, hypophyseal hormones and cholesterol may play a part in the maintenance or change of excitability of effector cells.

Summary. 1. Both the acetylcholine and potassium sensitivity of striated muscle and the activity of choline esterase of brain tissue were investigated in the presence of sex hormones and some of the hormones secreted by the hypophysis. 2. The acetylcholine sensitivity of the muscle is somewhat potentiated by the anterior pituitary-like sex hormone (Follutein, Squibb), the follicular hormone (Estrone Suspension, Abbott; Estradiol, Ciba; Theelin, Parke-Davis), the corpus luteum hormone (Progestin, Roche-Organon; Progesterol, Ciba), Testosterone Propionate (Ciba, Schering), the vasopressor and anti-diuretic hormone of the posterior part of hypophysis (Pitressin, Parke-Davis), but not by the oxy-

[9] Pompen, *De Invloed van Menformon op de Baarmoeder*, Theses, Amsterdam (cited by Reynolds, *Physiology of the Uterus*, Harper & Brothers, New York and London, 1939).

[10] Reynolds and Foster, *J. Pharm. Exp. Therap.*, 1940, **68**, 173.

[11] Reynolds and Foster, *Am. J. Physiol.*, 1939, **128**, 147.

tocic principle of the posterior part of hypophysis (Pitocin, Parke-Davis). 3. The activity of the choline esterase is somewhat decreased by Follutein, Estrone Suspension, Progestin, Testosterone Propionate, Pitressin, but not by Pitocin. 4. The potassium sensitivity of the muscle is potentiated by Follutein, Estrone Suspension, Estradiol, Theelin, Progestin, Progesterol, Testosterone Propionate, Doca (desoxycorticosterone, Roche-Organon), cholesterol, Pitressin, but not by Pitocin. 5. A correlation between threshold of excitability of the effector cells and the presence of the above substances was suggested.

The author wishes to express her gratitude to Abbott Laboratories, Ciba Pharmaceutical Products, Inc., Parke-Davis and Co., Roche-Organon, Inc., Schering Corporation, and E. R. Squibb and Sons for the generous supply of hormones.

14215 P

A Simple Method for the Concentration of Blood Plasma and Serum.

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In the course of the preparation of plasma for human use it was observed that on the thawing of frozen plasma, separation into a number of layers takes place; the lowermost deep-yellow layer is rich in proteins, while the uppermost, nearly colorless layer, contains only a small amount of protein. On repeated freezing and thawing, the separation becomes more distinct. A more rapid and sharp separation of plasma into layers is obtained by centrifuging the frozen material.

It is interesting that Bujwid[1] used the freezing method for the concentration of diphtheria and tetanus anti-toxin in 1897. His results were confirmed by Ernst, Coolidge and Cook.[2] Rossi[3] used the freezing and centrifuging procedures for fractionating plasma and other colloidal solutions, and Hati[4] used this technic for the concentration of complement, opsonins and anti-sera. In view of the above observations, the question arose as to whether these procedures might be employed for the concentration of plasma without excessive loss of solids.

The following is a representative fractionation and analysis of plasma: 100 cc were placed in a separatory funnel and frozen at —10°C; on partial thawing at 4°C, a deep yellow fluid was obtained with a colorless ice block floating on the surface. The freezing and thawing process was twice repeated. During the thawing which followed the third freezing, the melting fluid was collected directly into graduated cylinders. Three fractions were obtained in this manner. The first fraction of 25 cc was deep-yellow; the second

TABLE I.

	cc plasma vol.	% total protein	% albumin	mg% non-pro-N	mg% cholesterol	mg% chlorides	agglutinins
Original fraction	100	5.3	3.5	40	125	400	1:16
No. 1	25	15.0	9.3	83	255	1107	1:64
No. 2	17	6.5	4.3	40	185	382	1:32
No. 3	58	0.7	0.5	17	28	100	1: 8

1 Bujwid, *Z. f. Bakt.*, 1897, **22**, 287.

2 Ernst, Coolidge and Cook, *J. Boston Med. Soc.*, 1898, **2**, 166.

3 Rossi, G., *Arch. di Fisiologia*, 1904, **2**, 638.

4 Hati, S., *Z. f. Bakt.*, 1909, **48**, 203.

was 17 cc, and the third, derived from the colorless ice block, was 58 cc.

Quantitative determinations of proteins, albumin, chlorides, cholesterol and isoagglutinins were performed on the 3 fractions and on a sample of the original plasma. The results are presented in Table I. From these data, it can be seen that in the first fraction all the solid plasma constituents are contained in high concentration.

The percentage of each plasma constituent as compared with that of the original plasma are given in Fig. 1. From these, it is apparent that in the first fraction which represents only 25% of the original fluid volume, 70% of the proteins and chlorides is present, while in the third fraction, 58% of the original volume, only 8% of the proteins and 18% of the chlorides are present. Centrifugation of a frozen sample of plasma yielded similar results more rapidly and with a more distinct separation of the layers.

From the above findings, it is felt that these rapid and simple methods which do not require the use of chemicals may be of value in the concentration of plasma and serum constituents.

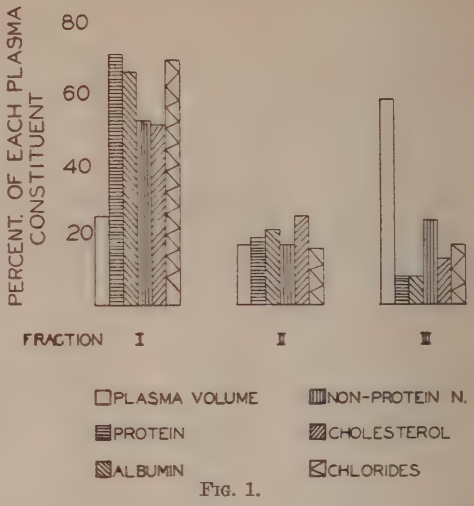

FIG. 1.

14216

Urinary Excretion of Acid-Decomposable Hydrocarbon Precursors Following Administration of Polycyclic Hydrocarbons.

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In 1934 it was shown[1] that when naphthalene is administered to rabbits there is excreted in the urine a compound which is decomposed by acid, but not by alkali, to yield naphthalene. Later work[2] revealed that such a compound is present in the urine of the rat and other species after the administration of naphthalene. Anthracene[3] and phenanthrene[4] when administered to rats and rabbits give rise to the excretion of compounds which yield anthracene and phenanthrene respectively when decomposed by acid. None of these hydrocarbon precursors has been isolated from urine. The present work was undertaken in order to obtain quantitative data on the excretion of these compounds and to study the question of whether analogous compounds are formed in the organism from other polycyclic hydrocarbons, namely, acenaphthene, chrysene, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene and methylcholanthrene.

An isolation procedure was developed in

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1 Bourne, M. C., and Young, L., *Biochem. J.*, 1934, **28**, 803.

2 Stekol, J., *J. Biol. Chem.*, 1935, **110**, 463; 1935, **113**, 675; 1937, **121**, 87.

3 Boyland, E., and Levi, A. A., *Biochem. J.*, 1936, **30**, 1225.

4 Young, L., and Britton, A., unpublished observations.

order to determine the amount of hydrocarbon liberated by the acid decomposition of urine obtained from rats after hydrocarbon administration. The sample of urine (50 ml) was made alkaline to litmus and extracted in a continuous extractor for 6 to 8 hours with ether (150 ml). This ether extract was evaporated and the residue was tested in order to determine whether it contained any acid-decomposable hydrocarbon precursor. With none of the hydrocarbons studied was the preliminary ether extract of the alkaline urine found to contain acid-decomposable hydrocarbon precursor. After the preliminary extraction with ether the urine was acidified with hydrochloric acid to a pH measured by a Coleman pH electrometer and allowed to stand at room temperature. The urine was then made alkaline to litmus with sodium hydroxide and re-extracted for 10 hours with ether (150 ml). This ether extract was washed first with 2N HCl, then with 2N NaOH, and finally with water. It was dried over anhydrous calcium chloride, filtered into a weighed flask and evaporated. The residue so obtained was dried in a desiccator and weighed. The weight of residue less the "blank value" for the urine of undosed rats represented the weight of hydrocarbon liberated by decomposition of the precursor compound. In a series of experiments with 50 ml samples of normal rat urine the average "blank value" was found to be 2.2 mg and in no experiment did it exceed 3.0 mg. In some of the experiments described later hydrocarbons were administered in liquid paraffin. Control experiments in which rats were dosed with liquid paraffin showed that the administration of this substance did not influence the "blank value" of the urine to a detectable extent. The efficiency of the isolation procedure was investigated in a series of experiments in each of which a known amount (10-50 mg) of hydrocarbon for which the procedure was to be used was added to a sample of rat urine which had previously been rendered alkaline and extracted with ether. Under these conditions the recovery figures obtained ranged from 94 to 102% of the amount of hydrocarbon added.

A study of the decomposition of the naphthalene precursor present in the urine of rats dosed with naphthalene showed that at room temperature the optimal acidity for the liberation of naphthalene was pH 1.5 to 2.5 and that under these conditions the amount of naphthalene liberated did not increase after 8 hours. Determinations were then made of the amounts of naphthalene liberated in periods of not less than 8 hours at pH 1.5 to 2.5 at room temperature from the urines of groups of male white rats dosed with known amounts of naphthalene. The rats used weighed from 200 to 300 g each. Two methods of administering the hydrocarbon were employed. In the first of these the hydrocarbon was dissolved in warm liquid paraffin and given by stomach tube. In the second method of administration the rats were fed for 2 one-hour periods each day in feeding cages on the stock colony diet to which the hydrocarbon had been added to the extent of 1%. Significant amounts of naphthalene precursor were not detected in the urine excreted later than two days after the completion of dosing. In order to determine the proportion of the dose of naphthalene which was excreted as the acid-decomposable hydrocarbon precursor, analyses were made of the urine collected during the dosing period and for three days after the completion of dosing. Experiments similar to those described above were also performed with phenanthrene and with anthracene. The results obtained in the experiments with naphthalene, phenanthrene and anthracene are summarized in Table I.

Experiments were performed in which 2 male rabbits, each weighing approximately 2 kg, were dosed with naphthalene. Each rabbit received by stomach tube a single dose of 2 g of naphthalene dissolved in warm liquid paraffin. The urine from each rabbit was collected daily for 3 days after the administration of the naphthalene, and the amount of naphthalene liberated from the urine under the optimal conditions described above was determined. The maximum liberation of naphthalene occurred in the urine collected on the first day after dosing. Traces of naphthalene were liberated from the urine excreted on the third day after dosing. In the urine collected in the 3-day period after dosing 5.9% of the naphthalene administered was isolated in the experi-

TABLE I.

Amounts of Hydrocarbon Liberated at pH 1.5-2.5 at Room Temperature in Periods of Not Less than 8 Hours from Rat Urine Excreted Following Administration of Naphthalene, Phenanthrene or Anthracene.

Hydrocarbon	No. of rats in group	Mode of administration	Mg of hydrocarbon admin.	Mg of hydrocarbon liberated	% of hydrocarbon intake liberated from urine
Naphthalene	8	Stomach tube	1600	224	14.0
"	8	" "	800	121	15.1
"	4	Mixed in diet	535	89	16.6
"	4	" " "	770	98	12.7
Phenanthrene	4	Stomach tube	800	48	6.0
"	4	" "	800	43	5.4
"	4	Mixed in diet	720	28	3.9
"	4	" " "	685	50	7.3
Anthracene	4	Stomach tube	800	18	2.3
"	4	" "	800	26	3.3
"	4	Mixed in diet	845	23	2.7
"	4	" " "	830	21	2.5

ment with one rabbit and 8.5% in the experiment with the other.

Experiments were also performed with the non-carcinogenic hydrocarbons acenaphthene and chrysene, and the carcinogenic hydrocarbons 3,4-benzpyrene, 1,2,5,6-dibenzanthracene and methylcholanthrene. These hydrocarbons were administered singly to groups of male white rats by admixture in the stock colony diet to the extent of 1%. The conditions of the experiments were similar to those described above in which naphthalene was administered to rats by this method. After the adequacy of the isolation procedure had been established for each hydrocarbon the urines were subjected to the conditions which were found to be optimal for the breakdown of the naphthalene precursor excreted by rats receiving naphthalene. The application of the isolation procedure to the acidified urines yielded small amounts of material which in every experiment corresponded to less than 1% of the hydrocarbon administered. In no case, however, when the residues from a series of experiments with a given hydrocarbon were combined and examined was it possible to detect the presence of hydrocarbon. In no experiment was hydrocarbon found on acidification of the residue obtained by evaporation of the preliminary ether extract of the alkaline urine, and the failure to isolate hydrocarbons from the acidified urines was therefore not due to removal of the precursor compounds by the preliminary ether extraction of the urines. It therefore appears that the urines of the rats dosed with acenaphthene, chrysene, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene or methylcholanthrene either did not contain hydrocarbon precursors decomposable under the conditions used or that such precursors were present in smaller amounts than could be detected by the methods employed.

Summary. After the administration of naphthalene to rats there is excreted in the urine a compound which can be decomposed by acid to yield naphthalene. The optimal acidity for the decomposition of this compound at room temperature was found to be pH 1.5-2.5, and under these conditions the amounts of naphthalene liberated did not increase after 8 hours. When the urines obtained after the administration of naphthalene to rats by stomach tube or by admixture in the diet were acidified under the optimal conditions described above, from 12.7 to 16.6% of the naphthalene administered was liberated. In similar experiments with phenanthrene and with anthracene the acidification of the urines obtained resulted in the liberation of from 3.9 to 7.3% of the phenanthrene and from 2.3 to 3.3% of the anthracene administered. No liberation of hydrocarbon was detected when the urines excreted by rats on diets containing acenaphthene, chrysene, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene or methylcholanthrene were acidified to pH 1.5-2.5. If

the ingestion of these hydrocarbons by rats is followed by the excretion of acid-decomposable hydrocarbon precursors either such compounds are excreted in very small amounts or their decomposition occurs under conditions which differ from those which bring about the breakdown of the naphthalene precursor excreted after the administration of naphthalene.

14217 P

Antigenic Relationships of the Species *Shigella dispar.*

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Castellani[1] isolated from the feces of patients with clinical dysentery a bacterium which he named *"Bacillus ceylonensis B."* Five years later[2] he reported the isolation from normal human stools of *Bacillus madampensis*. Both organisms were gram-negative, nonmotile rods, fermenting many carbohydrates, including lactose, with production of acid only, and producing indol. *Bacillus ceylonensis B* fermented dulcitol, whereas *Bacillus madampensis* did not. Both fermented sucrose. Andrewes[3] included these 2 species, together with some other lactose-fermenting dysentery-like varieties, in his *"Bacillus dispar."* Current terminology places these microörganisms in the genus *Shigella*.

Castellani stated that *Bacillus ceylonensis B* was a serologically homogeneous species. Glynn and Starkey,[4] working with 15 strains, reported *Shigella dispar* antigenically heterogeneous. The present investigation was undertaken to study further the antigenic structure of strains designated *Shigella dispar* or *Shigella ceylonensis*.

Thirty-seven strains of bacteria are included in this preliminary report. Five were received from the American Type Culture Collection; 10 were received from Dr. K. M. Wheeler, and were isolated in Connecticut within the past 4 years; 3 were isolated in Texas in 1942, and were received from Dr. MacDonald Fulton; two were isolated recently in Rhode Island by Dr. C. A. Stuart; the remaining 17 were isolated in a number of different laboratories along the Atlantic seaboard. Eleven strains of *Sh. ceylonensis* were received, and 26 strains of *Sh. dispar*.

All 37 strains produced acid from dextrose, lactose, maltose, and mannitol; failed to ferment salicin, and were indol positive. Further fermentation reactions are noted in Table I.

Antiserums were prepared for 2 strains of *Sh. dispar* and one of *Sh. ceylonensis*. Table I presents a summary of the results obtained when the 37 strains were tested for agglutination in the various antiserums. The titers stated are those for the type strains listed, but all organisms grouped together behaved similarly.

It is apparent that the 11 strains of *Sh. ceylonensis* are serologically identical or closely related to *Sh. dispar* type 205. Most of the type 205 strains were pigmented, the color varying from a faint yellowish-orange to a deep orange. None of the *Sh. ceylonensis* strains were pigmented, but there appears to be no correlation between pigment production and antigenic structure.

Two other antigenic types, 171 and 221, are evident. Thus, there are 3 well defined serological groups, the major group including 21 of the 26 strains of *Sh. dispar*, together with all 11 strains which were designated *Sh. ceylonensis* on the basis of their fermentation reactions.

This grouping of the strains is confirmed by adsorption experiments. Antiserums in 1:100 dilution were adsorbed twice for one hour at

1 Castellani, A., *J. Hyg.*, 1907, **7**, 1.

2 Castellani, A., *C. f. Bakt.*, 1912, I, Orig., **65**, 262.

3 Andrewes, F. W., *Lancet*, 1918, **194**, 560.

4 Glynn, J. H., and Starkey, D. H., *J. Bact.*, 1939, **37**, 315.

TABLE I.
Fermentation and Agglutination Reactions of *Shigella ceylonensis* and *Shigella dispar*.

Species	Type strain	Total strains	Fermentation of* Sucrose	Dulcitol	Xylose	Sorbitol	Rhamnose	Serum agglutination† Anti-167 (*ceylon.*)	Anti-205 (*dispar*)	Anti-171 (*dispar*)
Shigella ceylonensis	167	11	+	+	+	+	±	1:12,800	1:12,800	1:1,600
Shigella dispar	205	21	±	—	±	±	+	,,	,,	,,
	171	2	+	—	+	+	+	1:100	1:100	1:25,600
	221	3	—	±	±	+	+	<1:50	<1:50	<1:50

* + indicates acid production,
± indicates acid production by some strains.
† Tests were read after 12-18 hours at 55°C.

37°C with heavy antigenic suspensions. Some of the results are shown in Table II.

TABLE II.
Reciprocal Adsorption of Anti-*ceylonensis* and Anti-*dispar* Serums.

Antiserum	Adsorbing strain	Test strain	Agglutination titer × 100
167	Unadsorbed	167	1:128
	205	,,	<1:1
	171	,,	1:64
205	Unadsorbed	205	1:128
	167	,,	<1:1
	171	,,	1:128
171	Unadsorbed	171	1:256
	167	,,	1:256
	205	,,	1:256

It is evident that strains 167 and 205 are serologically identical. Since these cultures represent *Sh. ceylonensis* and *Sh. dispar* respectively, the conclusion seems warranted that these 2 names designate the same organism, differing only in pigmentation and in one or two biochemical characteristics. Type 171 (Tables I and II), while antigenically related to type 205 (*Sh. dispar*), possesses a major antigenic fraction not shared by type 205. On the other hand, type 221, biochemically related to *Sh. dispar*, appears to have no antigens in common with type 205. The present work confirms the antigenic heterogeneity of *Sh. dispar*, as shown by Glynn and Starkey; nevertheless, 86% of the 37 strains used in this investigation were antigenically closely related or identical.

Leaving in abeyance for the present the question of priority, it is tentatively suggested that bacterial groups designated *Shigella ceylonensis* and *Shigella dispar* be combined and defined as gram-negative, non-sporeforming rods which produce indol, ferment dextrose and lactose with production of acid only, and may or may not ferment sucrose or dulcitol.

14218

Vitamin E Deficiency in Rats Given Succinyl Sulfathiazole in Purified Diets.

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Daft, Ashburn and Sebrell[1] reported the occurrence of various lesions in rats given sulfaguanidine (sulfanilylguanidine) or sulfasuxidine (succinyl sulfathiazole) in purified diets. These lesions included hyalinization, necrosis and calcification of voluntary muscle. Ashburn, Daft, Endicott and Sebrell[2] remarked on the similarity of these lesions to those ascribed to vitamin E deficiency. They noted

[1] Daft, Floyd S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

[2] Ashburn, L. L., Daft, Floyd S., Endicott, K. M., and Sebrell, W. H., *Pub. Health Rep.*, 1943, **57**, 1883.

that the diets into which these sulfonamide drugs were incorporated were marginal or possibly deficient in vitamin E but that hyalinized or necrotic muscle fibers were seen but rarely in control rats. We wish to report at this time the occurrence of these muscle lesions (hyalinization, necrosis and calcification) in a group of rats given a somewhat similar purified diet containing sulfasuxidine and their prevention by α-tocopherol.*

Twelve litters of 3 rats each were placed on experiment at weaning at 21 to 25 days of age. Each group of litter-mates consisted of rats of the same sex. One rat in each litter was given experimental diet No. 735, which contains sulfasuxidine, and a weekly oral supplement of 3 mg of α-tocopherol (Merck) in ethyl laurate; another was given the same diet (No. 735) but no α-tocopherol supplement; and the third was given the control diet No. 736, which does not contain sulfasuxidine, and no α-tocopherol supplement. The control diet No. 736 consists of leached and alcohol extracted casein 25 g, sucrose 63 g, salt mixture 550[3] 4 g and lard 8 g, into which is incorporated 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 1 mg of pyridoxin hydrochloride, 4 mg of calcium pantothenate, 2 mg of niacin, 100 mg of choline chloride and 400 μg of 2-methyl-1,4-naphthohydroquinone diacetate (vitamin K). The experimental diet No. 735 is identical except that 1% of sulfasuxidine replaces an equivalent amount of sucrose. Twice weekly each rat was given an oral supplement of 0.25 cc of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola).

Twenty-two of the 24 rats receiving sulfasuxidine died after 36 to 84 experimental days. Eleven of these had been given the α-tocopherol supplement and 11 had not; their average survival times were 48 and 58 days, respectively. The remaining 2 animals receiving sulfasuxidine and 9 control animals were sacrificed at the end of 3 months, the other 3 controls at the end of 4 months on the experiment.

The incidence of muscle lesions is shown in Table I. These lesions have been described in detail previously.[2]

TABLE I.
Incidence of Muscle Lesions.

No. rats in group	Sulfasuxidine in diet	Individual weekly supplement of α-tocopherol	No. rats with muscle lesions
12	0	0	0
12	1%	0	9
12	1%	3 mg	0

From the negative findings with the control rats, it does not appear that the basal diet which we have employed will produce muscle damage in these young rats during the experimental period used. The high incidence of muscle lesions in the rats which were given sulfasuxidine in an otherwise identical diet appears to implicate this drug in their production while the preventive action of α-tocopherol indicates that these changes are due to a deficiency of vitamin E. It is not clear at the present time whether this deficiency arises through an increased demand for the vitamin, through interference with utilization of an otherwise adequate supply, or through a decrease in the amount available to the tissues.

While α-tocopherol is effective in preventing muscle lesions it evidently does not completely neutralize the toxic effects of sulfasuxidine as is evidenced by its failure to prolong the life of the experimental animal.

Conclusions. (1) Hyalinization, necrosis and calcification of voluntary muscle occur in rats given succinyl sulfathiazole in certain purified diets. (2) These lesions may be prevented by the oral administration of α-tocopherol.

* A purified diet, containing sulfaguanidine instead of sulfasuxidine but otherwise identical to one (No. 735) used in the present study has been fed for 3 months and has failed almost completely to bring about the occurrence of muscle lesions. The differences between our earlier[1] and present experimental conditions are under investigation.

[3] Spicer, S. S., Daft, Floyd S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep.*, 1942, **57**, 1559.

14219 P

Effect of Pituitary Adrenotropic Hormone on Lymphoid Tissue.*

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Introduction. This is a preliminary report of organ weight changes observed in studies initiated to investigate influences of adrenal cortical secretion on lymphoid tissue.

The existence of a reciprocal relationship between the size of the adrenal cortex and the thymus has been shown by previous workers.[1–6] Thymic atrophy in the rat following treatment with pituitary adrenotropic hormone has been reported.[7–10] It is also known that deficient adrenal secretion results in hypertrophy of the thymus.

Recently, pure adrenotropic hormone has been prepared,[11] making possible the study of effects of normal physiological stimulation of the adrenal cortex on the thymus. The paucity of data concerning the effects of adrenal cortical secretion on the mass of lymphoid tissue suggested careful studies of lymph nodes, spleens and thymi. The purpose in these researches was to investigate the normal mechanism of physiological control of lymphoid tissue.

Experimental. Mice of both sexes, 45 to 55 days old (CBA strain, Strong), were used in all experiments. One group of 5 animals were given daily subcutaneous injections of 0.5 mg adrenotropic hormone‡ in 0.5 ml aqueous solution. A second group of 18 animals were injected subcutaneously daily with 1.0 mg hormone in 0.5 ml.

In order to determine whether injection of protein *per se* influences lymphoid tissue, and to test effects of another pure anterior pituitary hormone, 6 mice were injected subcutaneously daily with an amount of prolactin[12] twice the highest dose of adrenotropic hormone used (2 mg prolactin in 0.5 ml solution).

In all experiments, mice were sacrificed at successive three-day intervals over a 15-day period.

A fourth group of 12 animals, litter-mates of the experimental ones, served as controls. These animals were sacrificed at various periods during the study.

The following tissues were dissected out carefully and weighed on a torsion balance: inguinal, axillary and mesenteric nodes, spleen and thymus. Other organs weighed were gonads, heart, adrenals and kidneys. Lymphoid tissues were saved for histological examination. Blood studies, bone marrow differentials and tissue histology will be presented at a later date.

Results. Data obtained for tissue weights are presented in Table I. Weights over the

* This investigation has been aided by grants from The International Cancer Research Foundation, the Fluid Research Fund, Yale University School of Medicine, and the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

† Fellow of the International Cancer Research Foundation.

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8 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 42.

9 Noble, R. L., and Collip, J. B., *Endocrinology*, 1941, **29**, 934.

10 Houssay, B. A., *J. Am. Med. Assn.*, 1942, **118**, 833.

11 Sayers, G., White, A., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 199.

‡ Acknowledgment is made to Mr. George Sayers for assistance in preparing the adrenotropic hormone used in this investigation.

12 White, A., Bonsnes, R. W., and Long, C. N. H., *J. Biol. Chem.*, 1942, **143**, 447.

TABLE I.
Effect of Adrenotropic Hormone on Weight of Lymphoid Tissue of the Mouse.

Exper.*	No. of animals	Tissue wts as mg/100 g of initial body wt†					
		Paired inguinal nodes	Paired axillary nodes	Paired mesenteric nodes	Thymus	Paired adrenals	Spleen
Controls	12	70.9± 3.4‡	56.1± 2.6	131.4±11.8	166.5±11.0	20.6±1.8	329±22
Adrenotropic hormone injected	23	52.3± 2.5	46.3± 2.9	89.6± 6.2	67.6± 7.8	30.8±1.0	370±15
Prolactin injected	6	53.5±23	59.4±18	99.2±26	175.6±74	19.5±6.4	326±17

* All animals received Fox Chow and water *ad libitum*.

† Data are expressed in terms of body weights of the mice at the beginning of the injection period. Thus, alterations in body weight resulting from injection of hormone do not influence the results.

‡ Means and standard errors.

TABLE II.
Significance of Average Values for Lymphoid Tissue Weights.

Tissue	P values* for adrenotropic hormone inj. animals	P values* for prolactin inj. animals
Inguinal nodes	<.01	.03
Axillary nodes	<.01	.6
Mesenteric nodes	<.01	.1
Thymus	<.01	.7
Adrenals	<.01	.7
Spleen	.06	.9

* P values as compared to the same tissues in control group.

15-day injection period are grouped together. The statistical significance of the differences between weights of tissues from control and hormone-injected animals have been calculated by the method of Fisher[13] for small series and are presented in Table II. It is evident from the tables that injection of adrenotropic hormone produced statistically significant decreases in weights of inguinal, axillary and mesenteric nodes, and in thymic mass. It can be calculated from Table I that the total weight of lymphoid tissue in the treated animals, exclusive of the spleen, is approximately half (2558 mg) that of similar tissue from control animals (4249 mg).

As expected, the adrenal weights of adrenotropic hormone injected animals are significantly greater than controls. Although the difference between the average weight of the spleens in the animals injected with adrenotropic hormone and in the control mice is not clearly statistically significant, the trend of the data suggest that spleen weight is increased in mice injected with adrenotropic hormone. Other organs weighed in this study showed no significant differences.

In prolactin-injected mice, reduction in weight of inguinal nodes occurred which is of questionable statistical significance. Other tissues from these animals showed no significant weight changes.

Although the relation of the adrenal cortex to the thymus has been the subject of previous investigations, the behavior of other lymphoid tissue under the influence of pituitary adrenal cortical stimulation has not been carefully examined. It seems scarcely necessary to emphasize the importance of knowledge concerning the factors controlling variations in lymphoid structures.

The failure of the spleen to follow the same weight trends as other lymphoid tissue may be better interpreted when histological studies are completed.

The results which have been obtained strongly suggest that activity of the adrenal cortex may have a profound influence on normal maintenance of lymphoid tissue.

Summary. Injection of pure pituitary adrenotropic hormone into CBA strain mice produces a decrease in weight of the inguinal, axillary and mesenteric nodes, and of the thymus. The spleen did not show a weight decrease.

[13] Fisher, R. A., *Statistical Methods for Research Workers*, 1932, 4th Ed., Oliver and Boyd, London.

14220 P

Isolation of an Iron Pigment From Human Red Hair.

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Since 1878 it has been known that a red substance can be extracted with mineral acids from human red hair.[1,2] This pigment is not obtainable from red hair of animals or from any human hair except bright red.

Experiments. In the authors' experiments red hair was treated with chloroform, acetone and glacial acetic acid respectively in the cold for fat removal, dehydration and initiating keratolysis. The colored matter was then completely extracted within 2 hours by boiling the hair with 0.1 N HCl in a reflux condensor. The color of the hair was not modified by this procedure.

The purplish-red clear extract (pH 1.5) turned brown on addition of alkali at pH 2.6 and regained its original red color on re-acidification. Further alkalinization precipitated the pigment at pH 7.0. Above this pH it again went into solution with a brown color. The sharp isoelectric point made the purification of the substance possible by repeated washing, dissolving, and re-precipitating.

The purified dry material, an amorphous dark red-brown powder with a metallic sheen, was insoluble in water, in organic solvents, and in acetic acid; it was easily dissolved by alkalies and by triethanolamine, a brown color resulting. Solution of the dry substance in acids was difficult but was easily accomplished after previous solution in alkali. The dry pigment kept indefinitely at room temperature. Heated, it decomposed without melting and left a reddish-brown ash. One hundred grams of hair yielded about 40 mg of pigment.

Qualitative analysis revealed iron in a complex form. The thiocyanate test for ferric ions was positive whether the hair had been extracted in the presence or absence of oxygen.

Red acid solutions showed a narrow absorption band with a maximum at 535 mμ. This band disappeared from the spectrum after alkalinization.

The pigment was dialyzable. In a cataphoresis experiment the color migrated to the anode at pH 9.16.

On addition of strong mineral acids or of an excess of thiocyanate, a brown substance precipitated, which was soluble in alkalies only. From the supernatant fluid of either precipitate another red substance could be precipitated at pH 7, showing the same properties as the original pigment except for a stronger thiocyanate reaction.

The diazo reaction and reactions for aldehyde, ketone groups, for tyrosine, catechol, pyrrol and indole nuclei were negative. The solutions did not show fluorescence in ultraviolet light.

Quantitative microanalysis of the purified pigment was performed on 2 samples from different groups of individuals.*

Purified Pigment.

	C	H	N	Ash
First sample	45.06	5.04	7.43	7.31%
Second sample	45.27	4.88	7.06	13.11 (Fe 9.17)%

In a 5-minute micro-Van Slyke experiment the pigment yielded 0.32% amino nitrogen.

Quantitative analysis of the red cleavage products gave these results:

Cleavage Products.

	C	H	N	Ash
Decomposition by HCl	32.05	3.91	7.74	26.46 (Fe 18.51)%
Decomposition by KSCN	33.97	3.93	5.65	31.41 (Fe 21.30)%

Despite the great difference in iron content

[1] Sorby, H. C., *J. Anthropol. Inst. of Great Britain*, 1878, **8**, 1.

[2] Arnow, L. E., *Biochem. J.*, 1938, **32**, 1281.

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the C:H:N ratio in the original material and in the KSCN cleavage product was almost identical: 7.5:10:1 versus 7.1:10:1. The brown precipitate formed during the HCl cleavage yielded C: 47.27, H: 5.32, ash: 2.90%.

The difficulty in calculating empirical formulas indicates we must further purify the material. For the original pigment the tentative formula is suggested: $(C_{15}H_{20}N_2O_9)_2Fe$.

Comment. The purplish-red color of the iron-pigment, the reversible color change with the pH and the type of the absorption spectrum[3] strongly suggest that it belongs in the group of complex phenolic iron compounds. In such compounds a single ferric ion is complexly bound to a varying number of phenolic radicals.[4] The data of the cleavage products indicate that during decomposition some phenolic groups separate from the iron, whereas others do not. Thus in the supernatant fluid new compounds of smaller molecular weight are present with all the properties of the original pigment (including similar absorption spectra) but containing more iron.

Phenolic iron compounds, however, are less stable than the pigment of red hair. The latter's greater stability may be explained by assuming that the phenolic OH-group is attached to a heterocyclic ring containing nitrogen. The negative reactions for pyrrol and pyridine do not exclude such a possibility because substitutions on these rings modify their reactivity. At present, no definite statement can be made regarding the chemical structure of the iron-containing pigment of red hair.

Summary. A red iron pigment was isolated from human red hair. The presented data indicate that it may be a complex phenolic iron compound in which the phenolic OH group is attached to a heterocyclic ring containing nitrogen.

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14221

Physiological Studies on Five Patients Following Ligation of the Inferior Vena Cava.

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The effects of ligation of the inferior vena cava of the experimental animal upon the circulation and functions of the parts drained by its tributaries have been studied by many.[1,2] If the ligation is below the renal veins, there are no apparent serious results. The inferior vena cava has been ligated relatively frequently in man.[3,4] It has been noted that when the ligation is below the renal veins, there is little disturbance experienced by the patients. It is the purpose of this report to record briefly a few physiologic observations made on 5 patients in whom the inferior vena cava was ligated by Dr. C. G. Collins of the Department of Gynecology and Obstetrics of Tulane Medical School.

Methods. The patients were studied several days after operation when they were sufficiently well to be moved from Charity Hospital to a laboratory specially equipped for such observations. These people were too ill to be moved from the hospital to the laboratory for study before operation.

1 Polkey, H. J., *Urol. and Cutan. Rev.*, 1929, **33**, 394.

2 Whittenbergr, J. L., and Huggins, C., *Arch. Surg.*, 1940, **41**, 1334.

3 Krotski, J., *Chirurg.*, 1937, **9**, 425.

4 Ochsner, A., and DeBakey, M., *New England J. Med.*, 1941, **225**, 207.

All observations were conducted in an air-conditioned room with a temperature of 75°F ± 1° and relative humidity of 50% ± 1%. The patients rested in bed for at least 30 minutes before any measurements were made. The room was constructed to reduce psychic disturbances to a minimum, being very much like one previously described.[5] All observations except the venous pressure, were made essentially without the patient's knowledge except for small parts of the apparatus that were connected to her. All 5 patients were females.

The following measurements were made:

1. *The rate of water loss* from the skin of the right index finger tip, right second toe tip, right pretibial region and volar surface of the right forearm was determined quantitatively by a method previously described.[6] The rate of water loss was determined first at a temperature and relative humidity of 75°F ± 1° and 50% ± 1% respectively. After measuring the rate of water loss under these environmental conditions for two 15-minute periods the temperature of the room was raised to 100°F ± 2° and the relative humidity to 75% ± 2%. These levels were reached within 15 minutes and the rate of water loss was measured for 2 more 15-minute intervals. The room temperature and humidity were then returned to the original levels.

2. *The volume of pulsations* of the right index finger tip and right second toe tip were measured quantitatively by the plethysmosphygmographic method of Turner.[7] These determinations were made after the subject had been resting another 30 minutes at the comfortable temperature and humidity of 75°F and 50% respectively.

3. *The venous pressure* in a vein of the dorsum of the foot and left antecubital area with the respective parts at the level of the heart was determined directly by a method previously described.[8] The venous pressure measurements were made after completing the other studies.

The above observations were made from 8 to 18 days after ligation of the inferior vena cava. In every instance, the patients were free from fever and other evidence of infection when the studies were performed. In 4 subjects the measurements were repeated at intervals indicated by Table I. Patient No. 1 could not be contacted for a follow-up study.

Results. The results are summarized in Table I.

The venous pressure was greatly elevated in the veins on the dorsum of the feet following ligation of the vena cava while the pressure in the antecubital veins remained normal. In no patient did the pressure return to normal, even as long as 10 months postoperatively.

All patients developed edema postoperatively along with the increase in venous pressure. In the 5 persons the edema was worse when they were up and about and only one (Patient No. 3) was clinically free from edema during any time of observations. None of them developed any cyanosis, erythema, petechiæ, pallor, paresthesia, tenderness, or hyperesthesia. Except for edema of the legs below the knees, the patients were clinically well.

There were no significant disturbances in water loss under a comfortable room environment, or under a hot humid one. There was an increase in the rate of water loss from the skin in a normal fashion when the room was made hot and humid.

The volume of pulsations of the toe tips decreased markedly in all 5 patients following ligation of the inferior vena cava. Even the maximum volume of pulsation of the toe tips was less than the mean volume of pulsations in the normal toe tip, the former being 3.5 cmm per 5 cc of toe tip and the latter 4.0 cmm per 5 cc of toe tip. In patient No. 5 the volume of pulsations in the toe tip returned to normal by the time of the second observation, although the venous pressure was still abnormally high. There was no edema, however.

Discussion. Increase in venous pressure in the veins of the dorsum of the feet following ligation of the inferior vena cava is known to occur. The fact that the levels reached were

[5] Neumann, C., Cohn, A. E., and Burch, G. E., *J. Clin. Invest.*, 1942, **21**, 651.

[6] Neumann, C., Cohn, A. E., and Burch, G. E., *Am. J. Physiol.*, 1942, **132**, 748.

[7] Turner, R. H., *J. Clin. Invest.*, 1937, **16**, 777.

[8] Burch, G. E., and Sodeman, W. A., *J. Clin. Invest.*, 1939, **18**, 31.

TABLE I.

Venous Pressure, Rate of Water Loss from the Skin, and Volume of Pulsations of Peripheral Blood Vessels in Five Female Adults in Whom the Inferior Vena Cava Was Ligated Below the Renal Veins. Values in Normal Subjects Are Also Indicated.

Subject	Date	Age	Color	Venous pressure, cm H_2O			Rate of water loss mm³/15 min/10 cm²								Vol. of pulsations cc³/5 cc part		
							Rt. Ind. Fngr.		Rt. 2nd Toe		Rt. Leg		Rt. Forearm		Rt. Ind. Fngr. Tip	Rt. 2nd Toe Tip	
				Ante-cubital	Dorsum Rt. foot	Dorsum Lt. foot	C.T.	H.H.T.	C.T.	H.H.T.	C.T.	H.H.T.	C.T.	H.H.T.			
(1)	4-11	21	W	12.2		24.8	50.2	149.0	18.8	39.5			11.2	63.0	3.5	0.3	1
(2)	5-20	31	C		36.0	50.0+	21.6	98.7	7.7	30.1	11.0	30.6			4.0	0.5	2
	6-24			10.6	35.6	36.6	15.5	69.0	10.2	25.2	10.0	31.2			11.2	1.2	
(3)	5-22	21	W	11.0	41.6	50.6	16.5	30.8	13.0	16.9			9.8	16.0	6.9	3.5	3
	to 3-9			14.0	20.0	22.0	20.7	53.5	9.8	23.7			4.6	12.6	2.1	0.8	
(4)	4-20	67	W	2.6		24.4	15.5	27.7	6.9	19.5	18.0	38.2			8.9	1.2	4
	6-30			10.6		30.6	17.4	31.8	6.1	12.8	6.6	28.0			10.3	2.7	
	11-7			5.0	30.0	30.0	24.7	28.0	13.5	17.6	10.0	27.2			10.1		
(5)	5-7	27	W	14.0		29.0									4.1	0.4	5
	6-1			12.6		25.6	13.7	24.5	12.1	19.3			10.8	23.6	7.5	6.0	
	11-10			10.0	17.0	18.0	21.7	56.3	12.3	30.9			13.6	29.8	9.9	5.6	
	Mean			10.3	30.0	31.1	21.8	56.9	11.0	23.6	11.1	31.4	10.0	29.0	7.1	2.2	
	Max.			14.0	41.6	50.6	50.2	149.0	18.8	39.5	18.0	38.2	13.6	63.0	10.3	6.0	
	Min.			2.6	17.0	18.0	15.5	27.7	6.1	16.9	6.6	27.2	4.6	12.6	2.1	0.3	
Normal Values:																	
	Mean			8.0*	10.5†		23.2‡	47.9	12.5	31.1	8.3	32.1	4.4	25.9	6.9§	4.0§	
	Max.			14.0	11.0		41.3	52.1	15.7	48.7	13.6	37.6	8.4	37.8	12.4	11.5	
	Min.			3.0	10.1		14.0	21.7	10.4	21.8	4.1	22.4	2.0	14.0	0.9	0.7	

a = Mean values calculated from the data obtained on the first observations.

C.T. = Comfortable temperature of room (75°F and 50% relative humidity).

H.H.T. = Hot and humid temperature of room (100°F and 75% relative humidity).

* Normal values from Burch, G. E., unpublished data.

† Normal values from McIntire, I. M., and Turner, A. H., *J. Clin. Invest.*, 1935, **14**, 16.

‡ Normal values for all rates of water loss from Burch, G. E., and Sodeman, W. A., *Am. J. Physiol.*, 1943, **138**, 603.

§ Normal values from Burch, G. E., Cohn, A. E., and Neumann, C., *Am. J. Physiol.*, 1942, **136**, 433.

1 Inferior vena cava and both ovarian veins ligated on 3-19-42. Very slight pitting edema 3 weeks post-operatively. No edema on walking.

2 Thrombophlebitis of the left femoral vein extending into iliacs. Inferior vena cava, left femoral and both ovarian veins ligated on 5-2-42. Marked edema of both legs, especially left on 5-20-42. On 6-24 little edema; more marked when up and about. Died Nov. '42, suddenly outside N.O. No autopsy obtained.

3 Inferior vena cava and both ovarian veins ligated on 4-30-42. Marked edema of right leg and moderate edema of left leg on 5-22. 3-9-43, fatigue. No edema or other symptoms.

4 Inferior vena cava ligated on 3-28-42. Marked bilateral edema, especially of the right leg on 4-20 and 6-30-42. 11-7-42, asymptomatic with pitting edema of both legs, particularly the right.

5 Inferior vena cava ligated on 4-25-42. Mild edema on 5-7-42 and no edema on 6-1-42. 11-10-42, edema only when up a long time.

not greater indicates a good collateral circulation around the ligated vena cava. To a large extent the level of the venous pressure reached is inversely related to the degree of collateral circulation. The pressure did not return to normal in any of the patients, even after approximately 10 months following operation. It is possible that in some instances the venous pressure will eventually reach normal.

Every patient developed edema of the legs and feet postoperatively and only one (Patient No. 3) became clinically free from edema during the period of study. The increases in venous pressure were sufficient to produce edema. Patient No. 3 was clinically free from edema even though the venous pressure was still elevated indicating adequate compensating phenomena.

The rate of water loss from the surface of the skin of the tip of the right second toe and the pretibial area was not changed materially by ligation of the inferior vena cava. There was a normal increase in rate of water loss when the patients' environment was made hot and humid. An increase in venous pressure for several days apparently does not significantly influence water loss through the skin. No observations were made immediately after ligations of the vena cava.

The effects of ligation of the inferior vena cava upon the volume of pulsations in the tip of the right second toe is in keeping with previous observations.[9,10,11] It was found that an increase in venous pressure, produced by chemical irritation, ligation of the femoral vein, or by the influences of gravity, such as lowering the parts in man below heart level,[11] resulted in a marked decrease in the volume of pulsations of the distal blood vessels. The relationships between the increase in venous pressure and volume of pulsations were studied in a mechanical model and were found to be inversely related functions.[10] The effects of the decrease in the volume of pulsations in the peripheral blood vessels upon the efficiency of circulation, lymph flow, flow of intercellular fluids and the like remain unknown. Some studies indicated a decrease in the rate of lymph flow with a decrease in the volume of pulsations of blood vessels.[12]

Clinically these 5 patients compensated remarkably well following ligations of the inferior vena cava. The sudden death of patient No. 2, about 6 months following operation, may have been related to the ligation. The clinical history regarding her sudden death suggested hemorrhagic encephalitis following an intravenous injection of arsenic for lues as the cause. An autopsy could not be obtained.

Summary. The venous pressure, rate of water loss from the skin and volume of pulsations in the peripheral blood vessels were measured in 5 patients following ligation of the inferior vena cava below the level of the renal veins.

The venous pressure increased markedly in the veins of the dorsum of the foot. Edema developed immediately after operation and only one patient was edema-free approximately 10 months after operation. In no patient did the venous pressure in the veins of the foot return to normal up to 10 months postoperatively.

The rate of water loss from the skin of the tip of the right second toe and pretibial area was normal by the eighth postoperative day. There were no measurements earlier.

The volumes of pulsations of the peripheral blood vessels were decreased markedly by ligation of the inferior vena cava.

All patients showed a remarkably good compensation of the circulation following ligation.

9 DeBakey, M. E., Burch, G. E., and Ochsner, A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 585.

10 Burch, G. E., DeBakey, M., and Sodeman, W. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 858.

11 Turner, R. H., Burch, G. E., and Sodeman, W. A., *J. Clin. Invest.*, 1937, **16**, 789.

12 Cressman, R. D., and Blalock, A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 140.

14222

Selective Permeability of Skin Capillaries.

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The extensive experimental work on the so-called blood-brain barrier has shown that at least one area of the capillary system, namely, the capillaries of the central nervous system, is endowed with a selective permeability. As has been shown elsewhere,[1] these capillaries are permeable to electropositive and electroneutral substances, impermeable to electronegative ones. These conclusions were based on experiments with toxins, viruses, antibodies, drugs and aniline dyes in rabbits and guinea pigs. The experiments here reported are concerned with the permeability of the cerebral capillaries to aniline dyes in other species and with the permeability of the capillaries of other organs in guinea pigs.

Barbour and Abel[2] reported that after removal of the fore-brain of frogs the rest of the brain was stained by some sulfonated aniline dyes. We, therefore, investigated the permeability of the cerebral capillaries to basic and acid dyes in the intact frog. Frogs weighing approximately 50 g (*Rana pipiens*) were used and the dyes injected into the abdominal lymphsac. The results are recorded in Table I.

It will be seen that the results are different from those obtained in rabbits and guinea pigs. The cerebral capillaries of the frog are permeable to both basic and acid dyes while the cerebral capillaries of rabbits and guinea pigs are selectively permeable to basic dyes.

Vital staining experiments with some acid aniline dyes reported in the literature[1] mostly without references to our problem seem to indicate that the capillaries in some areas of the vascular system are permeable to acid dyes. The number of organs thus far investigated, however, is limited and the distribution of basic dyes has not been studied systematically.

Four ml of 1% solutions of a variety of basic and acid aniline dyes, therefore, were injected intravenously into guinea pigs weighing 300 g. The staining effects were observed macroscopically and are recorded in Table II.

As may be seen from Table II, muscles, epidermis, lungs, liver, kidneys, and adrenals are stained by both basic and acid dyes although not by all of them. The inner surface of the abdominal skin—and the same probably holds true for connective tissue in general—however is selectively stained by acid dyes.

At first sight this result might be explained by a selective affinity of connective tissue for acid aniline dyes. *In vitro* experiments, however, did not support this explanation. In

TABLE I.
Minimal Staining Doses of Basic and Acid Aniline Dyes in mg, in Frogs (*Rana pipiens*) Weighing 50 g.

Basic dyes		Acid Dyes	
Nile Blue	3.0	Eosin	8
Chrysoidine	3.2	Trypan Blue	9
Bismark Brown	6.5	Naphthol Yellow	10
Malachine Green	8.6	Acid Fuchsin	10*
Pyronine	68.0 (faint)	Congo Red	35 (faint)
Neutral Red	180.0 (faint)	Tropaeolin 00	No staining
Methylene Blue	No staining†		

* After acidification with hydrochloric acid.
† Even after acidification and treatment with H_2O_2.

1 Friedemann, U., *Physiol. Rev.*, 1942, **22**, 125.

2 Barbour, H., and Abel, J. J., *J. Pharm. and Exp. Therap.*, 1910, **2**, 163.

TABLE II.
Distribution of Basic and Acid Aniline Dyes in Various Organs.

	Inner surface of abdominal skin	Muscles	Epidermis	Lungs	Liver	Kidneys	Adrenals
			Basic Aniline Dyes.				
Methylene Blue	0	+	+	+	+	+	+
Night Blue	0	—	—	—	—	—	—
Brilliant Green	0	+	+	+	+	+	+
Chrysoidine	0	+	+	+	0 (?)	0	+
Nile Blue	0	+	—	+	—	0	—
			Acid Aniline Dyes.				
Trypan Blue	+	+	+	+	+	+	+
Indigodisulfonate	+	+	+	0	0	0	0
Light Green	+	+	—	0	0	—	+
Eosin	+	+	+	+	—	—	+

0 = No staining. + = Organ stained. — = Not investigated.

these experiments small bits of freshly excised abdominal skin from which the thin muscular layer covering the inner surface was removed were put into varying dilutions of basic and acid dyes in guinea pig serum. The staining effect is recorded in Table III.

TABLE III.
In vitro Experiments. All Dilutions of Dyes Made in Guinea Pig Serum.

Dilution of dye	Eosin	Methylene Blue	Trypan Blue	Night Blue
1:500	—	—	+	—
1:1,000	+	+	±	+
1:2,000	+	+	0	+
1:4,000	+	+	0	+
1:8,000	±	+	0	±
1:16,000	0	±	—	0
1:32,000	0	0	—	0
1:64,000	0	0	—	0
Cv	1:8,000	1:16,000	1:1,000	1:8,000

As may be seen from Table III basic dyes stain the skin even in higher dilutions than do acid dyes. The objection may be raised that the staining properties of the abdominal skin may undergo a rapid change after removal from the animal body. Transplantation experiments with skin, however, show that excised skin retains its viability during the time consumed in our *in vitro* experiments. Also, respiration of excised tissues remains intact for many hours. There is no reason, therefore, for supposing that the staining properties of the abdominal skin *in vitro* are essentially different from those in the intact animal. Nevertheless, this objection was met by the following experiment: In the anesthetized living guinea pig a flap of abdominal skin was formed by severing the skin in the middle line and along Pouparts ligament. The thin muscle layer on the peritoneal surface of the skin was removed with a forceps and drops of various dilutions of methylene blue and eosin in guinea pig serum placed on the inner surface of the abdominal skin at some distance from the cuts. Both dyes stained in a dilution of 1:10,000.

The *in vitro* experiments do not necessarily express any affinity of the dyes for connective tissue. Primarily probably both dyes penetrate the skin by way of diffusion. There is no reason why they should not do so from the vascular system provided the capillaries of the skin were perfectly permeable to both basic and acid aniline dyes. On this assumption the minimal staining concentration in the blood plasma (Cb) should be identical with the one observed *in vitro* (Cv). It will be shown presently that Cb can be calculated by dividing the intravenous minimal staining amounts of the individual dyes through the plasma volume (1/25 of the body weight). As a matter of fact, according to Wittgenstein and Krebs[3] basic aniline dyes leave the vascular system much more rapidly than do acid dyes. In previous investigations on the permeability of the cerebral capillaries,[4,1] however, it was shown that within the limits of experimental error Cb and Cv were identical for basic dyes. This result shows that the staining effect is determined by the initial

[3] Wittgenstein, A., and Krebs, H. A., *Pflügers Arch.*, 1926, **212**, 268, 282.

[4] Friedemann, U., *J. Immunol.*, 1937, **32**, 97.

TABLE IV.

In vivo experiments. All dyes injected intravenously in 1% solution. Animal killed 3 minutes after injection, by ether anesthesia.

	Wt of guinea pig in g	Dye in mg	Concentration in blood plasma	Result	Cb
I. Eosin	340	11	1:1,200	+	1:2,400
	460	7.6	1:2,400	±	
	300	2.5	1:4,800	—	
II. Trypan Blue	425	28	1:600	+	1:600
	375	12.5	1:1,200	—	
III. Methylene Blue	325	44	1:300	—	>1:300
	300	20	1:600	—	
IV. Night Blue	225	30	1:300	—	>1:300
	265	18	1:600	—	

Plasma volume is calculated by dividing the body weight of the experimental animal by 25. + = Strongly colored. ± = Slightly colored. — = Uncolored.

TABLE V.

Cb, Cv, and Cb/Cv.

	Cb	Cv	Cb/Cv
Eosin	1:2,400	1:8,000	3.3
Trypan Blue	1:600	1:1,000	1.7
Methylene Blue	>1:300	1:16,000	>53
Night Blue	>1:300	1:8,000	>27

concentration of the dye in the blood plasma. Cb, therefore, was calculated for eosin, trypan blue, methylene blue and night blue and the results are recorded in Table IV.

The Cb, Cv, and Cb/Cv values for the 4 dyes are recorded in Table V.

As may be seen from Table V the Cb and Cv values for acid dyes are not identical but very close to each other. On the contrary, the Cb/Cv values for basic dyes are far above 1. These experiments show that the capillaries of the skin are permeable to acid dyes, impermeable to basic dyes.

It may be emphasized that the colloidal or non-colloidal character of the dyes is apparently of no significance. For there are colloidal and non-colloidal dyes in both groups. The permeability of the cerebral capillaries was likewise found to be independent of the degree of dispersion of the dyes. The decisive factor is apparently the electrical charge. We thus reach the conclusion that both the cerebral capillaries and those of the skin (and probably of the connective tissue in general) are endowed with a selective permeability to polar substances. But one is the reverse of the other. The theoretical aspects of these findings will be dealt with in a separate paper.

14223

Comparative Solubilities of Sulfadiazine, Sulfamerizine and Sulfamethazine and Their N_4-Acetyl Derivatives at Varying pH Levels.

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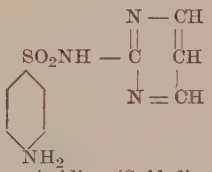
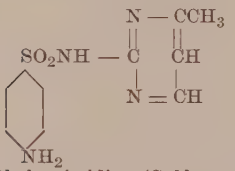
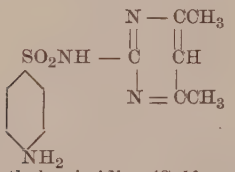
Monomethyl and dimethyl derivatives of sulfadiazine—namely, sulfamerizine and sulfamethazine—are currently receiving clinical trials.[1,2,3,4]



2-S*-pyrimidine (Sulfadiazine[6]†).



2-S-4-methylpyrimidine (Sulfamerizine[7]†).



2-S-4,6-dimethylpyrimidine (Sulfamethazine[8]†).

In vitro and *in vivo* studies have revealed that the bacteriostatic activities against several different organisms of these derivatives of sulfadiazine are quantitatively similar to those of sulfadiazine[6,9,10] and of sulfapyridine.[8]

It has been suggested that renal complications due to precipitation of the drugs or their acetyl derivatives‡ might be less frequent during therapy with sulfamerizine[3,4,7] and with sulfamethazine,[1,8] than with sulfadiazine. The different solubility characteristics of the three 2-sulfanilamidopyrimidine drugs and of their acetyl derivatives are therefore of particular interest. The solubilities of all of these compounds in buffer solutions of pH values within the physiological range of urinary pH are reported here; the findings for sulfadiazine and acetylsulfadiazine have also been presented elsewhere.[11]

Methods of Study. An excess of the compound§ to be dissolved was shaken in M/15 phosphate buffers and in M/10 citrate + NaOH buffers of various pH values for 18 hours in a water bath at 37°C and filtered in an incubator room at 37°C. The pH of the

1 Macartney, D. W., Smith, G. S., Luxton, R. W., Ramsay, W. A., and Goldman, J., *Lancet*, 1942, **1**, 639.

2 Jennings, P. A., and Patterson, W. H., *Lancet*, 1942, **2**, 308.

3 Goodwin, R. A., Jr., Peterson, O. L., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 262.

4 Murphy, F. D., Clark, J. K., and Flippin, H. F., *Am. J. Med. Sci.*, 1943, **205**, 717.

* S = Sulfanilamido; nomenclature by Crossley *et al.*[5]

† References to articles giving nomenclatures.

5 Crossley, M. L., Northey, E. H., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1938, **60**, 2217.

6 Roblin, R. O., Jr., Williams, J. H., Winnek, P. S., and English, J. P., *J. Am. Chem. Soc.*, 1940, **62**, 2002.

7 Welch, A. D., Mattis, P. A., Latven, A. R., Benson, W. M., and Shiels, F. H., *J. Pharm. and Exp. Therap.*, 1943, **77**, 357.

8 Rose, F. L., Martin, A. R., and Bevan, H. G. L., *J. Pharm. and Exp. Therap.*, 1943, **77**, 127.

9 Roblin, R. O., Jr., Winnek, P. S., and English, J. P., *J. Am. Chem. Soc.*, 1942, **64**, 567.

10 Bell, P. H., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, 1942, **64**, 2905.

‡ Throughout this report, "acetyl derivative" refers to the N_4-acetyl derivative.

11 Gilligan, D. R., Garb, S., and Plummer, N., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 248.

§ The sulfonamide compounds were supplied by Lederle Laboratories, Inc.

filtrate was measured immediately at room temperature with a Beckman glass electrode pH meter and appropriate corrections for the differences between room temperature and 37°C were applied. The amounts of dissolved compounds were measured according to the method of Bratton and Marshall.[12] Studies of the amounts of the drugs and of their acetyl derivatives dissolved in M/15 PO_4 buffer of pH 6.98 after 18 and 42 hours of shaking at 37°C showed that saturation had been reached in 18 hours. The results were calculated in terms of molar concentrations and sulfadiazine equivalents in mg per 100 cc for the purpose of ease of comparison. (Fig. 1, 2).

[12] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

Results. The solubilities of all of the compounds studied increase with increasing pH over the range of 5.0 to 8.0 (Fig. 1, 2). The solubilities in citrate buffers accord with those in phosphate buffers.

Both in terms of molar concentrations and weights of compounds dissolved per unit volume the acetyl derivatives of sulfadiazine and of sulfamethazine are more soluble than the parent drugs, respectively, throughout the range of urinary pH (Fig. 1,2). Similarly acetylsulfamerizine is more soluble than is sulfamerizine in terms of weight of compound dissolved; in terms of molar concentration, however, these two compounds have practically the same solubilities between pH 5.2 and 6.4 (Fig. 1, 2).

Sulfamethazine is the most soluble of the 3

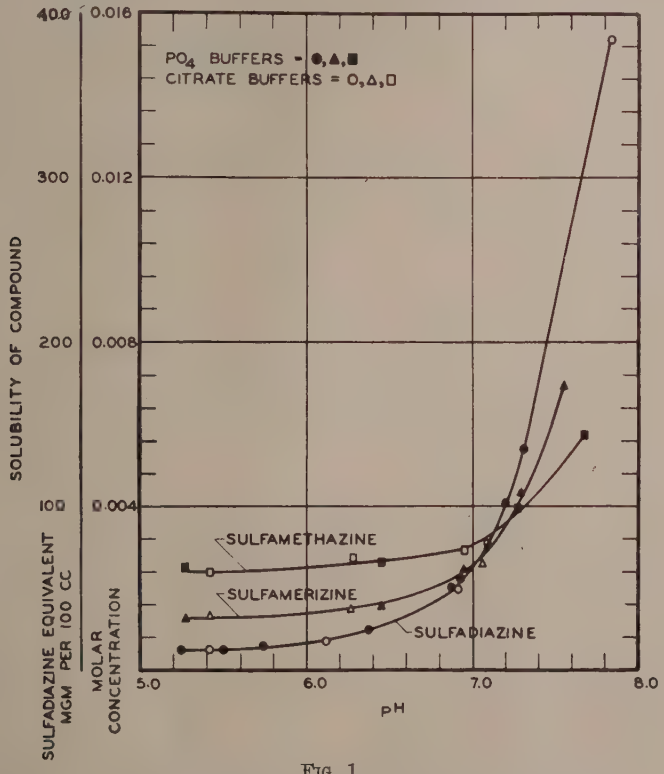


FIG. 1.
Curves of Solubility of Sulfadiazine, Sulfamerizine, and Sulfamethazine in Buffer Solutions.

The solid circles, triangles and squares represent measurements in phosphate buffers for sulfadiazine, sulfamerizine, and sulfamethazine, respectively. The open symbols represent measurements in citrate buffers.

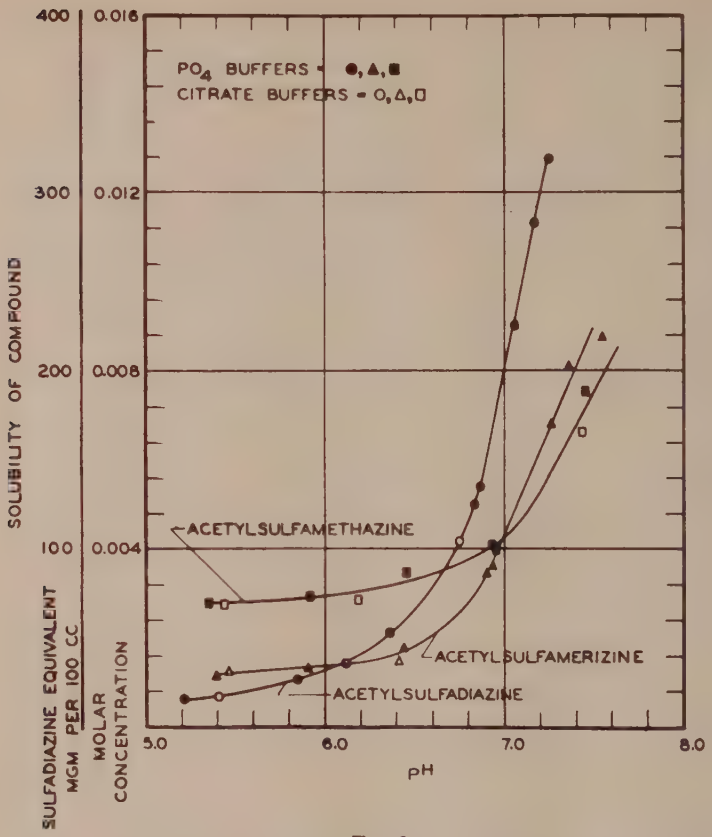


FIG. 2.
Curves of Solubility of Acetylsulfadiazine, Acetylsulfamerizine, and Acetylsulfamethazine in Buffer Solutions.
The solid circles, triangles and squares represent measurements in phosphate buffers for acetylsulfadiazine, acetylsulfamerizine, and acetylsulfamethazine, respectively. The open symbols represent measurements in citrate buffers.

drugs in the acid range of urinary pH, and sulfadiazine is the most soluble in the alkaline range (Fig. 1). Acetylsulfamethazine is more soluble than acetylsulfamerizine in the whole acid range of urinary pH and is more soluble than acetylsulfadiazine up to pH 6.65; acetylsulfadiazine is the most soluble of the 3 compounds in the alkaline range (Fig. 2).

Discussion. The data presented here accord in general with other recently published findings on the solubilities of some of these compounds in normal urine of varying pH,[7,13] or in H_2O plus varying amounts of NaOH.[1,8] It is to be noted that Rose *et al.*[8] and Macartney *et al.*[1] reported solubility data for "sulfamethazine hemihydrate," a meta-stable compound with lower melting point (178°-180°C) and much higher solubility[1,3] than those of sulfamethazine (Fig. 1). This "sulfamethazine hemihydrate" was the original sulfamethazine preparation produced by Imperial Chemical Ltd. (England), and which they subsequently have been unable to reproduce.[9] The sulfamethazine compound now produced

[13] Jensen, O. J., Jr., and Fox, C. L., Jr., *J. Urol.*, 1943, **49**, 334.

‖ This information was gained through a courteous personal communication, dated April 19, 1943, from Drs. H. R. Martin and F. L. Rose of Imperial Chemical, Ltd.

and distributed for clinical use[14] by them has the same melting point (197°-198°C) as that (198°-199°) produced for Lederle Laboratories, Inc., and used in our studies. We were able to study a sample of this later sulfamethazine product of Imperial Chemical Ltd., and found its solubility at varying pH levels to be the same as that of the sulfamethazine (Fig. 1) from Lederle Laboratories, Inc.

It was found recently that no crystalluria or renal complications occurred in a series of over 150 patients who had received sulfadiazine together with sufficient adjuvant alkali therapy to maintain the urine neutral or alkaline.[11,14] These clinical findings accord with the markedly increased solubilities of sulfadiazine and acetylsulfadiazine in alkaline solutions (Fig. 1, 2). Whether the incidence of renal complications from sulfamerizine or sulfamethazine will be sufficiently low to warrant the use of these drugs with no adjuvant alkali therapy awaits answer from further clinical trials. The daily amounts of the drug excreted in the urine on appropriate therapeutic doses, together with the extent to which the drug in the urine is acetylated, are factors of great importance. Since the urinary precipitates from all of the sulfonamide drugs are usually chiefly composed of the acetyl derivatives of the drugs,[14,15,16] it is of particular interest to note that the mean solubility of acetylsulfamerizine in the acid range of urinary pH is no greater than that of acetylsulfadiazine, whereas the mean solubility of acetylsulfamethazine in the acid range is considerably higher than that of acetylsulfadiazine (Fig. 2).

Summary. Comparative data are presented on the solubilities of sulfadiazine, sulfamerizine and sulfamethazine and their N_4-acetyl derivatives in buffer solutions at varying pH levels within the physiological range of urinary pH. The findings have been discussed briefly in reference to their possible bearing on renal complications from therapy with these drugs.

[14] Gilligan, D. R., Garb, S., Wheeler, C., and Plummer, N., *J. A. M. A.*, in press.

[15] Stewart, J. D., Rourke, G. M., and Allen, J. G., *J. A. M. A.*, 1938, **110**, 1885.

[16] Prien, E. L., and Frondel, C., *J. Urol.*, 1941, **46**, 748.

14224 P

Blood Lactic Acid in Liver Glycogen Disease.

Howard H. Mason and Grace E. Sly. (Introduced by R. McIntosh.)

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An increase in the amount of circulating lactic acid is generally attributed to anoxia.[1] This is the cause of the high values obtained during muscular work, in cardiac decompensation, anemia, pneumonia, and shock. The concentration of lactic acid in the blood can, however, be raised above the normal fasting level by the injection of epinephrine.[2] Cori[3] has demonstrated that in this case the lactic acid comes largely from the breakdown of muscle glycogen. This phenomenon is apparently not associated with anoxia.

We have recently had the opportunity to observe 2 infants with a singular disease, in which the high blood lactic acid would seem to be due to some other mechanism than that postulated. The disease in question, commonly known as hepatic glycogen disease or von Gierke's disease, is characterized by a defect in liver metabolism, in consequence of which the liver accumulates abnormally large stores of glycogen which it is unable to transform into glucose and excrete into the blood as a normal liver does during hypoglycemia.

[1] Jervell, O., *Acta Med. Skand.*, 1928, **24**, 1.

[2] Loeb, R. F., *J. Clin. Invest.*, 1931, **10**, 19.

[3] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1928, **79**, 309.

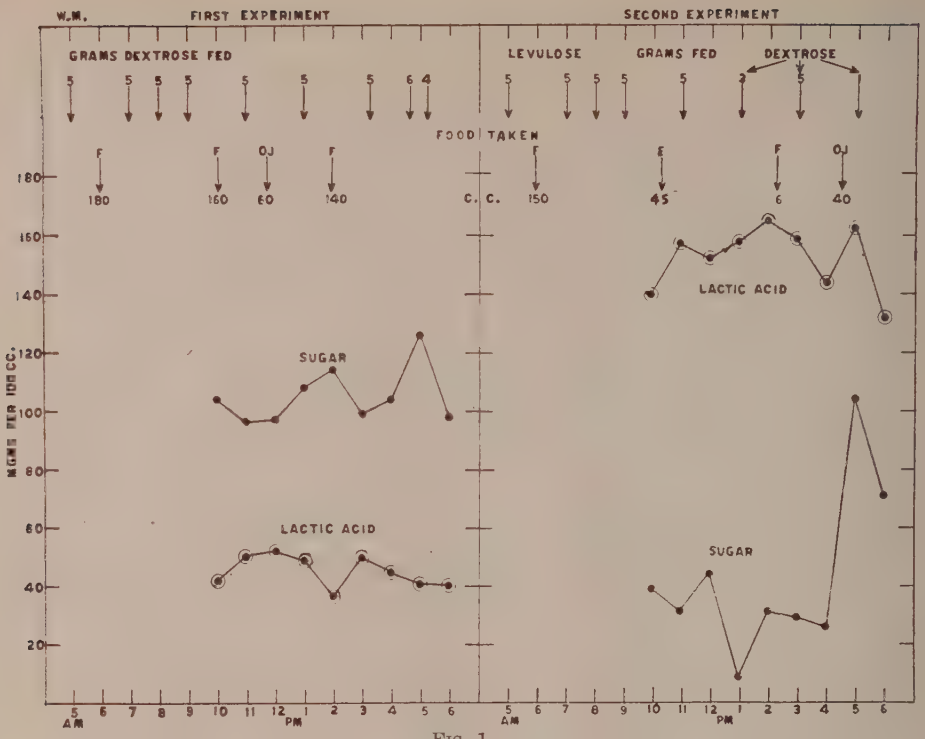

FIG. 1.

Blood sugar and lactic acid on 2 days. The blood lactic acid is nearly 3 times as high on the levulose day as on the glucose day. The feedings (F) were the same on both days. Each 100 cc contained soy bean flour 6 g, soy bean oil 3.2 g, glucose 1.8 g, sucrose 0.9 g, maltose 0.7 g, dextrine 1.2 g. Orange Juice (OJ) which contains 12% levulose, was fed on each day. On one day 5 g of glucose and on the other 5 g of levulose was offered each hour between the regular feedings. After 3 P.M. on the levulose day, glucose was fed because the infant refused the levulose and showed signs of hypoglycemic shock. This accounts for the sharp rise in blood sugar at the end of the observation.

The patients are female infants; one 6 months, the other 6½ months old. Both have very large livers. Neither shows any rise in blood sugar after the injection of epinephrine. Both tend to have very low blood sugar if not fed more frequently than a normal infant. Both have had convulsions due to hypoglycemia. Simultaneous determinations* of blood sugar and lactic acid have been made 110 times. An analysis of the correlation of these individual determinations shows no consistent relation between the height of the blood sugar and that of the lactic acid. When, however, these infants are fed soy bean mixture and corn syrup at the usual 4-hourly intervals and also given 30 to 50 cc of a 10% glucose solution every hour between feedings, the blood sugar stays at normal or slightly higher levels and the lactic acid remains at between 20 and 50 mg per 100 cc (Fig. 1). If levulose is substituted for glucose in the inter-

* All determinations were made on capillary blood. The skin was thoroughly washed with alcohol, then with ether. The first drop of blood was discarded. After precipitation of the proteins with tungstic acid,[4] sugar was determined on the filtrate by Benedict's[5] method and lactic acid by the method of Barker and Summerson.[6]

[4] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry, II. Methods*, Baltimore, Williams and Wilkins Co., 1932, 461.

[5] Benedict, S. R., *J. Biol. Chem.*, 1931, **92**, 141.

[6] Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

vals between the regular feedings, the blood sugar remains much lower but the lactic acid reaches and maintains an abnormally high concentration.

In an individual with a normal liver both monosaccharides help to maintain the blood sugar at a normal level. In glycogen storage disease of the liver the two sugars behave differently. Absorbed levulose is quickly removed from the portal blood by the liver and stored there as glycogen or fat, but cannot be released later as glucose to sustain the falling blood sugar. Glucose, on the other hand, is removed with difficulty from the portal blood by the liver;[7] therefore, during the absorption of glucose the blood sugar rises to a high concentration and then falls rather rapidly, due to the removal of glucose from the blood by tissues other than the liver for combustion or storage. The only way to raise the low blood sugar in the postabsorptive state is to feed more glucose. This explains the hypoglycemia when the extra sugar fed is levulose and the normal or high blood sugar when the extra sugar is glucose. It does not explain the high blood lactic acid after the blood sugar has been low for some time, nor the much lower blood lactic acid after the blood sugar has been normal for a few hours.

Under the conditions of the test the lactic acid content of the blood is only slightly more than normal as long as the blood sugar is within the normal range, but increases several fold when the blood sugar is maintained at an abnormally low level. Neither patient shows any of the recognized causes of anoxia, nor any of the symptoms that characterize this condition, nor does either patient show any symptoms of hyperadrenalinemia.

The inference would appear to be that prolonged hypoglycemia causes the breakdown of liver glycogen in the cases reported as it does in the livers of normal individuals. However, in von Gierke's disease the liver is unable to dephosphorylate hexosemonophosphate and discharge glucose into the blood. Blocked at this point the breakdown takes the alternate route, namely, levulose-6-phosphate to levulosediphosphate, etc.[8] In this case one of the end products would be lactic acid.

[7] Mason, H. H., and Andersen, D. H., *Am. J. Dis. Child.*, 1941, **61**, 795.

[8] Cori, C. F., *Biol. Symposia*, 1941, **5**, 131.

14225

Effect of *p*-Aminobenzoate and Methyl *m*-Amino-*p*-hydroxybenzoate on Acute Toxicity of *m*-Amino-*p*-hydroxyphenylarsenoxide in Mice.

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p-Aminobenzoic acid (PAB) has been shown by Sandground[1] to antagonize the single-dose fatal toxicity of a number of pentavalent organic arsenicals in rats. That this antagonism was probably not due alone to phenomena dependent on close structural similarity was indicated by the fact that acetarsone (sodium *m*-acetylamino-*p*-hydroxyphenylarsonate) was one of the arsenicals antagonized. More recently, and coincidental with the completion of the work reported here, Sandground[2] reported success in antagonizing the toxicity of "at least one of the trivalent arsenical antisyphilis drugs" with PAB; the identity of the compound was not disclosed, however.

The toxic signs observed by Sandground, and which were attributable to the pentavalent arsonic acids antagonized by PAB, were of the neurotropic type characteristic of this class of compounds. Since it is clear that some

[1] Sandground, J. H., *Science*, 1943, **97**, 73.

[2] Sandground, J. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 188.

reduction to the analogous arsenoxide derivatives takes place *in vivo*,[3] the ability of PAB to antagonize the toxicity of the widely used antisyphilitic arsenical compound, *m*-amino-*p*-hydroxyphenylarsenoxide ("Mapharsen," Parke, Davis Co.), has been studied. Also, the ability of *m*-amino-*p*-hydroxybenzoic acid, as its methyl ester ("Orthoform," Winthrop Chemical Co.), to perform a similar role has been investigated.

Procedure. Martin and Thompson[4] found the antagonistic effect of cysteine and ascorbic acid to the acutely toxic effects of arsphenamine in mice to be most pronounced when the antagonists were administered several hours before the arsenical. A similar procedure was employed in these experiments in order to allow adequate time for the diffusion of PAB and methyl *m*-amino-*p*-hydroxybenzoate, Orthoform, throughout the tissues.

White mice of both sexes were used, PAB and Orthoform were given in doses which were experimentally demonstrated to be definitely sublethal. PAB was given as a solution of the sodium salt (pH 6.8 to 7.2) and Orthoform a suspension in 0.1% mucilage of tragacanth. Controls given a tragacanth suspension without drug showed no ill effects and the toxicity of Orthoform in this vehicle and in olive oil was the same. Tragacanth was chosen for convenience of administration. The Mapharsen was injected as an aqueous solution into the tail vein.

Results. Deaths began to occur in mice within one to 2 hours following an intravenous injection of lethal doses of Mapharsen; the majority of deaths occurred during the first 24 hours. No deaths occurred after 72 hours although the mice were observed for 10 days. The results are shown in Table I.

The concentration of free PAB in the blood of a number of mice was determined by the sulfonamide method of Marshall and his coworkers,[5,6] using *p*-toluenesulfonic acid as the protein precipitant.

Discussion of Results. The small difference in mortality rates in Experiment 1 and 4 is not considered significant. Although larger and equal numbers of animals would be required fully to substantiate this opinion, no antagonism of significance could be represented by such a small decrease in mortality. Furthermore there was no difference in the time of death of mice receiving Mapharsen alone and in those receiving Mapharsen plus PAB. The data on the concentration of PAB

TABLE I.
Acute Toxicity of Mapharsen in Mice, with and without the Previous Administration of PAB or Orthoform.

Exp.	Mapharsen per kg	PAB per kg	Orthoform per kg	Interval between doses	Deaths in 24 hours No.	%	Total deaths No.	%
1	35.0 mg	0 g	0 g		15/20	75	16/20	80
	"	1 i.p.	0	2 hr	12/19	63	13/19	68
	"	0	0.5 i.p.	2 "	10/16	62	12/16	75
2	"	0	0		13/17	76	14/17	82
	"	2 i.p.	0	1½ "	12/16	75	13/16	81
3	32.5 mg	0	0		13/30	43	16/30	53
	"	0	0.5 i.p.	1 "	20/28	71	25/28	89
4	35.0 mg	0	0		15/18	83	15/18	83
	"	1 oral	0	1½ "	11/15	73	11/15	73
5	"	0	0		11/15	73	11/15	73
	"	1 i.p.	0	1 min	12/15	80	12/15	80

[3] Murgatroyd, F., Russell, H., and Yorke, W., *Ann. Trop. Med. Parasit.*, 1934, **28**, 227.

[4] Martin, G. H., and Thompson, M. R., *Exp. Med. Surg.*, 1943, **1**, 38.

[5] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

[6] Marshall, E. K., Jr., Litchfield, J. T., and White, H. J., *J. Pharm. and Exp. Therap.*, 1940, **69**, 89.

TABLE II.
Concentration of PAB in the Blood of Mice Following the Administration of Various Dosages of PAB.

Dose per kg	Time after admin., hr	PAB in blood	Avg
1 g i.p.	2	107, 131, 221, 113, 120	138
2 g i.p.	1½	446, 453, 382, 442,	431
1 g oral	1½	127, 125, 121, 110, 159	128

in the blood (Table II) indicate that a high concentration of PAB was present when the arsenical was injected. Even larger doses of PAB have been injected intraperitoneally (3g/kg), but also without the production of significant antagonism of Mapharsen toxicity; at this dosage level PAB caused occasional delayed deaths and higher doses could not be employed.

The data presented in Table I indicate that Orthoform exerts no protective action against Mapharsen toxicity. An explanation for the higher mortality rate with Orthoform in one experiment is not apparent. When Orthoform was given intravenously as a solution of the hydrochloride (pH 2.8) (175 mg/kg), prior to Mapharsen, 10 of 21 mice died (48%), while 15 of 26 (54%) which received dilute hydrochloric acid of the same pH, prior to Mapharsen, died without a significant difference in the survival time of the 2 groups.

Summary. No significant reduction in the acutely fatal toxicity of *m*-amino-*p*-hydroxyphenylarsenoxide (Mapharsen) in mice was produced by the prior administration of sodium *p*-aminobenzoate or the methyl ester of *m*-amino-*p*-hydroxybenzoic acid (Orthoform) under the conditions employed.

Grateful acknowledgment is made to Miss Helen Morrison for technical assistance, and to Miss Ethol Shiels and Miss Elizabeth Patch for the determination of PAB concentrations in blood.

14226

Treatment of Experimental Renal Hypertension With Vitamin A Concentrates.*

G. E. WAKERLIN AND W. G. MOSS.

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Subsequent to Govea-Peña and Villaverde's[1,2] favorable results in the treatment of essential hypertension in man with large doses of vitamin A orally, we obtained significant reductions in the blood pressures of renal hypertensive dogs with a vitamin A concentrate.[3] In our preliminary report, however, we indicated our intention of investigating the possibility that the observed therapeutic effect of the concentrate was due to some unknown constituent of the concentrate other than vitamin A. We now report our results to date with vitamin A concentrates in experimental renal hypertension.

Methods. Seven dogs were rendered hypertensive by the Goldblatt technique and the resulting hypertension was permitted to stabilize over a period of 5 to 8 months. Mean blood pressure readings were obtained by puncture of a femoral artery 2 to 3 times a week. Studies on the blood urea nitrogen, urinalyses, and determinations of body

* Aided by a grant from the Winthrop Chemical Company, New York City.

1 Govea-Peña, J. and Villaverde, M., *Rev. Cubana Cardiol.*, 1940, **2**, 332.

2 Villaverde, M., and Govea-Peña, J., *Bol. de la Aso. Med. de P. R.*, 1941, **33**, 238.

3 Wakerlin, G. E., Moss, W. G., and Smith, E. L., *Science*, 1942, **96**, 161.

weight were made at monthly or bi-monthly intervals. Three dogs were treated daily with 200,000 units of Lot 1 of a vitamin A concentrate† dissolved in 1 cc of sesame oil by mouth for 3 months, followed by 400,000 units of the concentrate in 2 cc of sesame oil for an additional 3 months. These dogs have been observed for 6 to 9 months since treatment was stopped. One dog was given a daily dose of 400,000 units of a highly purified vitamin A alcohol in 2 cc of sesame oil orally for 3½ months, and after a rest period of 2 months, 400,000 units of Lot 2 of the vitamin A concentrate in sesame oil daily for 1½ months, followed by the same dosage of Lot 3 of the concentrate in fish liver oil for 1½ months. One dog received 2 cc of Lot 4 of the concentrate in fish liver oil subsequent to heat inactivation of the vitamin A in the presence of oxygen, daily by mouth for 3½ months, followed by a rest period of 2 months and then by 400,000 units of Lot 2 of the concentrate for 1½ months and the same dosage of Lot 3 of the concentrate for 1½ months. The remaining 2 dogs were given oral daily doses of 1 cc of sesame oil for 3 months, followed by 2 cc of sesame oil for another 3 months. One of these 2 animals has been observed for 7 months subsequent to sesame oil administration. The other, after a rest period of 4 months, received 400,000 units daily of Lot 2 of the concentrate for 1½ months and the same dosage of Lot 3 for 1½ months.

In addition to the therapeutic experiments and in view of the prophylactic effect of certain renal extracts in experimental renal hypertension,[4,5] 6 dogs were given 400,000 units of Lot 2 of the vitamin A concentrate daily by mouth for 3 months before and 3 months after constriction of the renal arteries in order to test the possible prophylactic effect of the concentrate. Five control dogs were given oral daily doses of 2 cc of sesame oil for 3 months before and 3 months after constriction of the renal arteries. Otherwise these animals were subjected to the same observations as the dogs on the therapeutic experiments.

Blood serum vitamin A and carotene determinations were made on the dogs at intervals of 2 to 4 weeks by the Carr-Price method as modified by McCoord and Luce-Clausen.[6]

Results. I. *Therapeutic Experiments.* Striking reductions in blood pressure were observed in each of the 3 dogs given Lot 1 of the vitamin A concentrate. The results for one of the dogs which are typical for the other 2 animals are illustrated in Fig. 1. The blood pressures of this dog were reduced from a hypertensive range of 190-210 mm Hg. to the preconstriction normotensive level of 130-140 mm Hg. Since treatment was stopped 6 months ago, the blood pressures have shown a gradual increase to 160-180 mm Hg. In each dog there was a significant decrease in blood pressure during the third and fourth weeks of treatment with a more gradual fall during the following 5 months of therapy.

The dog given the purified vitamin A and later Lots 2 and 3 of the concentrate showed no significant change in its hypertensive level during or subsequent to treatment. This was likewise true for the dog receiving the heat-inactivated vitamin A concentrate and later Lots 2 and 3, and also for the 2 dogs given sesame oil, including the one subsequently given Lots 2 and 3 of the concentrate.

II. *Prophylactic Experiments.* Three of the vitamin A concentrate dogs and 2 of the sesame oil dogs developed typical experimental hypertension following moderate constriction of the renal arteries. The remaining 3 concentrate dogs and 3 sesame oil dogs developed malignant hypertension terminating fatally in one week or less subsequent to severe constriction of the renal arteries.

III. *Vitamin A Determinations.* Serum vitamin A determinations on the dogs not receiving vitamin A showed values of 25-270

† The vitamin A concentrates and other preparations studied were supplied through the courtesy of the Department of Medical Research, Winthrop Chemical Company, New York City.

4 Wakerlin, G. E., Johnson, C. A., Smith, E. L., Moss, W. G., and Weir, J. R., *Am. J. Physiol.*, 1942, **137**, 515.

5 Goldblatt, H., *J. A. M. A.*, 1943, **122**, 136.

6 McCoord, A. B., and Luce-Clausen, E. M., *J. Nutrition*, 1934, **7**, 557.

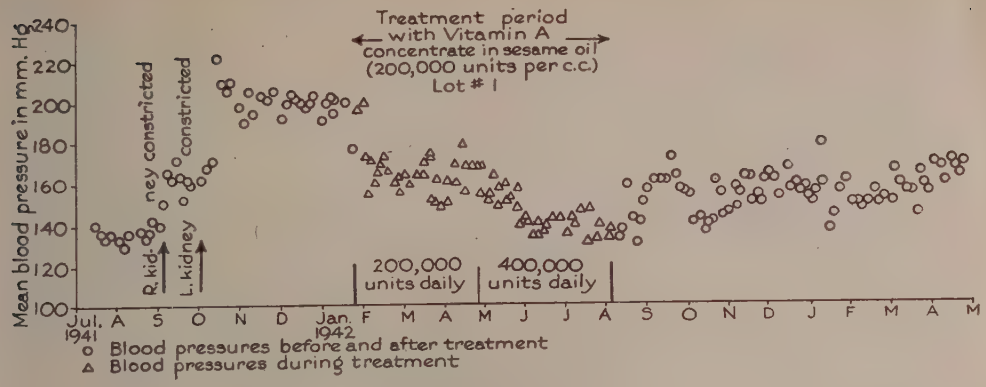

FIG. 1.

γ/100 cc. During vitamin A administration, the determinations varied between extremes of 250 and 8300 γ/100 cc. The variations in any single dog, however, were less pronounced. During the months following vitamin A administration the serum values have decreased but slightly. The carotene values varied from 0 to a trace.

IV. *Absence of Toxic Effects.* No toxic effects were detected in any of the animals. The appetites of the dogs remained excellent, their weights constant, and their blood urea nitrogens and urines normal. The two dosages of vitamin A used were somewhat less than 1/20 and 1/10 of the amounts reported toxic for rats by some workers[7,8,9] but less than 1/100 and 1/50 of the toxic levels reported by others[10,11] who contend that the lower values of the former investigators are due to impurities.

Discussion. The group of 3 hypertensive dogs treated with Lot 1 of the vitamin A concentrate is admittedly small but the antihypertensive effect in each animal was striking. We have never seen spontaneous blood pressure decreases similar to the reductions observed in these 3 dogs in 100 renal hypertensive animals during the past 3 years. Nor did a number of reputedly antihypertensive agents studied by one of us lower the blood pressures of renal hypertensive dogs.[12]

Obviously the antihypertensive effect of Lot 1 of the concentrate was not due to vitamin A inasmuch as Lots 2 and 3 and the purified vitamin A alcohol were inactive antihypertensively. Moreover, Katz and his coworkers obtained no antihypertensive effects in renal hypertensive dogs from a different vitamin A concentrate[13] or from Lot 2 of the concentrate studied by us.[14] Likewise, Geiger[15] found no significant blood pressure change in one hypertensive dog given still another type of vitamin A concentrate. Inasmuch as Lot 4 of the concentrate was not tested for antihypertensive activity prior to heating, no conclusions can be drawn from the negative results with the heat-inactivated vitamin A concentrate. Before we determined that Lots 2 and 3 were without antihypertensive effect, we had interpreted this result as suggesting that the antihypertensive activity of Lot 1 would be destroyed by heating in the presence of oxygen.

Since Lot 2 of the concentrate subsequently proved to be without therapeutic antihypertensive effect, its failure to have any prophy-

7 Domagk, G., and von Bobeneck, P., *Virch. Arch. f. Path. Anat.*, 1933, **290**, 385.

8 von Drigalski, W., *Klin. Woch.*, 1933, **12**, 308.

9 Popper, H., and Brenner, S., *J. Nutrition*, 1942, **23**, 431.

10 Vedder, E. B., and Rosenberg, C., *J. Nutrition*, 1938, **16**, 57.

11 Ikegaki, I., *Ztschr. f. Vitaminforsch.*, 1938, **7**, 113.

12 Wakerlin, G. E., and Gaines, W., *Am. J. Physiol.*, 1940, **130**, 568.

13 Katz, L. N., *J. A. M. A.*, 1943, **122**, 60.

14 Katz, L. N., personal communication.

15 Geiger, E., personal communication.

lactic effect is not surprising.

At present we have no explanation for the marked difference in the results obtained with Lot 1 and Lots 2 and 3 which presumably were prepared by the same method. Possibly some unrecognized variation in the method of preparation led to the production of the unknown antihypertensive constituent in Lot 1. Unfortunately further studies with Lot 1 can not be conducted since the supply is exhausted.

The relationship of the antihypertensive substance in Lot 1 to that reported by Grollman and Harrison[16] in fish liver oils is, of course, problematical. The vitamin A concentrate which they reported as showing some therapeutic effect in hypertensive rats was the same as Lot 2 which proved inactive in our hypertensive dogs.

We are of the opinion that an exhaustive search for the antihypertensive agent of Lot 1 is well-warranted, since it was effective by mouth and apparently in small amounts.

This report should discourage the use of vitamin A concentrates in the treatment of human hypertension, unfortunately stimulated by our preliminary report.

[16] Grollman, A., and Harrison, T. R., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 162.

Conclusions. 1. One lot of a vitamin A concentrate dissolved in sesame oil produced striking reductions in the blood pressures of renal hypertensive dogs when administered by mouth in a dosage of 200,000 units daily for 3 months followed by 400,000 units daily for 3 months.

2. Second and third lots of the concentrate dissolved in sesame oil and fish liver oil respectively, a purified vitamin A alcohol in sesame oil, and a fourth lot of the concentrate dissolved in fish liver oil and subjected to heat inactivation of the vitamin A, as well as sesame oil, were all without antihypertensive effect. Likewise the second lot of the concentrate showed no prophylactic effect in experimental renal hypertension.

3. No toxic effects were observed in the dogs.

4. Obviously the antihypertensive effect of the first lot of the concentrate was not due to vitamin A but to some unknown constituent not present in the second and third lots.

5. Since this antihypertensive substance was effective by mouth and apparently in small amounts, further work leading to its reproduction and identification is highly desirable.

14227

Supra-Diaphragmatic Section of the Vagus Nerves in Treatment of Duodenal Ulcer.*

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Much of the experimental work on the pathogenesis of gastroduodenal ulcer that has appeared in recent years is in harmony with the concept that the cause of these ulcers is the corrosive action of the gastric juice. It has been adequately demonstrated that pure gastric juice can destroy and digest living tissues including the wall of the stomach itself, producing in this case a defect which appears to be identical with the lesion encountered in man.[1] Under normal conditions, the gastric wall is not digested away apparently because it is not exposed to pure gastric juice. Food, which in the normal individual is the stimulus for the formation of gastric juice, is also the chief factor which protects the tissues against

* This work has been aided by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

[1] Dragstedt, L. R., *Arch. Surg.*, 1942, **44**, 438.

its corrosive activity. Pancreatic juice, gastric and intestinal mucus, duodenal juice and bile (probably in the order named) constitute an additional mechanism which protects the duodenal and, to a certain extent, also the gastric and the jejunal mucosa. When excessive volumes of normal gastric juice are continuously secreted in experimental animals, this defensive neutralizing mechanism is overcome, and ulcer is produced. Wangensteen and his associates[2] have produced ulcers in many experimental animals by the implantation of pellets of histamine mixed with beeswax into the muscles or beneath the skin. The gradual liberation of histamine provoked a long continued secretion of gastric juice. Most ulcer patients display an excessive secretion of gastric juice in response to the stimulus of food, histamine, or alcohol. A considerable number secrete large amounts of gastric juice when there is no obvious stimulant, as at night when the stomach has been previously emptied of food by lavage. The cause of this abnormal secretion is unknown. Presumably it might be due to the continuous formation of histamine-like substances in the body or to the constant excessive activity of the gastric secretory fibers in the vagus nerves. It was to secure information on this latter possibility that the present work was done.

Supra-diaphragmatic section of the vagus nerves was performed on 2 patients with duodenal ulcer. The left side of the chest was opened after removing the eighth rib. The lower esophagus was mobilized for a distance of about 10 cm. Vagus fibers were readily identified, separated from the esophagus by blunt finger dissection and collected in two main bundles. Segments of these nerves, 3 to 4 cm in length were excised, the ends ligated, and the pleura closed. The chest was closed without drainage.

The first patient was a male, age 51, who was first seen in December, 1938. He had been operated upon for perforated duodenal ulcer in 1930. He remained well until 1938, when he again developed epigastric distress, relieved by food and alkalies. X-rays showed a crater in the duodenum and blood appeared in the stools Medical management was carried out with partial success until December, 1942, when the symptoms again recurred. At this time he was found to secrete large volumes of highly acid gastric juice at night. A small rubber tube was introduced through the nose into the stomach. At 9:00 P.M. the stomach was emptied and continuous suction maintained to collect the gastric secretion for the following 12 hours. Sleep was not interfered with. Aspirations were carried out 13 times usually with a rest period of one or 2 days between tests. An average of 1160 cc of gastric juice was obtained, the minimum being 800 and the maximum 1600 cc. The pH of the juice varied between 1.19 and 2.98 with most of the samples between 1.45 and 1.70. Section of the vagus nerves was performed January 18, 1943, and following the operation aspiration was carried out 9 times. The volume of secretion obtained fell to an average of 310 cc, the minimum being 162 and the maximum 615 cc. The pH varied between 2.0 and 6.7 with most of the samples between 2.36 and 3.20. In April 2 aspirations were made yielding 162 and 375 cc with a pH of 3.2 and 6.3 respectively. The ulcer symptoms have been relieved and no disturbance in swallowing has appeared.

The second patient was also a male, age forty, who was first seen in March, 1940. A non-stenosing duodenal ulcer was diagnosed by the symptomatology, x-ray findings, and direct visualization of the crater by the gastroscope. A massive hemorrhage occurred in June, 1942. In February, 1943, aspiration of the 12 hour gastric content at night was carried out 6 times. The volume of gastric juice obtained varied between 990 and 1700 cc with an average of 1450 cc. The pH of the secretion varied between 1.11 and 1.40. Section of vagus nerves was done on February 22, 1943. In March, aspiration of the night secretion was carried out 6 times, the volume secured varying between 150 and 750 cc with an average of 510 cc. The pH varied between 1.5 and 3.9. In April, 1943, 3 aspirations were made, the amounts secured being 550,

2 Walpole, S. H., Varco, R. L., Code, C. F., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 619.

350, and 300 cc and the pH 3.9. This patient also has been relieved of his epigastric distress and has experienced no difficulty in eating.

The data obtained from these 2 patients indicates that the excessive night secretion of gastric juice in ulcer patients is probably neurogenic in origin and may be markedly reduced by supra-diaphragmatic section of the vagus nerves. Although both of these patients appear to have been benefitted by the operation, it is obvious that the period of observation is too short and the number of patients too small to warrant recommendation of this procedure at this time for the treatment of duodenal ulcer.

14228 P

Evaluation of the Ratio of Aortic Rigidity to Peripheral Resistance.

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The expressiveness of pulse rate and blood pressure measurements may be enhanced by introducing the values obtained into the formula described below. This formula helps to differentiate between the effects which conditions in different parts of the cardiovascular system produce upon the blood pressure by offering a number which expresses the ratio of aortic rigidity (or capacity) to the peripheral resistance.

Various investigators (most recently Apéria[1]) in the field of vascular dynamics (comprehensively reviewed by Wezler and Böger[2]) have evaluated the E and W functions, and the E/W ratio. E is the volume-elasticity coefficient and is a measure of the rigidity (or capacity) of the aorta and other large arteries (*windkessel*) and W is a measure of the peripheral resistance. (These symbols are adopted from Wezler and Böger). The evaluations of this ratio published hitherto have been based on data obtained by technics which are not readily available in most laboratories and clinics, as for instance measurements of cardiac output. It is the purpose of this note to point out that the E/W ratio is independent of the stroke volume of the heart, and that this ratio may be determined from heart rate and blood pressure measurements alone.

Development of the Formula. E (volume-elasticity coefficient) is defined as $\frac{\Delta P}{\Delta V}$, where ΔP is the pulse pressure, and ΔV is the change in volume of the *windkessel* corresponding to the given ΔP. Wezler and Böger have equated ΔV to $\frac{V_s}{2}$, where V_s is the stroke volume of the heart. E then becomes $\frac{\Delta P}{\frac{1}{2}V_s}$. In absolute units, E has the dimensions $\frac{\text{dynes}}{\text{cm}^5}$.

W (peripheral resistance) is defined as $\frac{P_m{}^*}{V_s \cdot r/60}$, where P_m is the mean arterial pressure, V_s is the stroke volume as before, and r is the heart rate per minute. The denominator of the ratio is the second-volume of the heart. In absolute units, W has the dimensions $\frac{\text{dynes} \cdot \text{seconds}}{\text{cm}^5}$.

The ratio E/W therefore is equal to $\frac{\Delta P}{\frac{1}{2}V_s} \div \frac{P_m}{V_s \cdot r/60}$, which simplifies to $\frac{r \cdot \Delta P}{30 P_m}$. It should be noticed that V_s cancels out. The constant (30) depends upon the use of Wezler

[1] Apéria, A., *Acta Physiologica Scandinavica*, 1941, **2**, 64.

[2] Wezler, K., and Böger, A., *Ergeb. Physiol.*, 1939, **41**, 292.

* The venous pressure theoretically should be subtracted from P_m in the numerator of the ratio, but ordinarily may be neglected because of its small magnitude compared with P_m.

TABLE I.
The Effects of Epinephrine upon the Value of E/W. (Data from Grollman,[3] after Euler and Liljestrand.)

	Pulse rate	Blood pressure	Pulse pressure	Mean pressure	E/W
Before injection	60	103/68	35	86	0.82
After inj. of 0.7 cc 1:1000 epinephrine subcut.	66	130/64	56	92	1.34

TABLE II.
The Effects of Pitressin upon the Value of E/W. (Data from Grollman,[3] p. 196.)

	Pulse rate	Blood pressure	Pulse pressure	Mean pressure	E/W
Before injection	67	102/70	32	86	0.83
After inj. of 0.4 cc pitressin intramusc.	64	107/81	26	94	0.59

TABLE III.
The Effects of Pyrotherapy upon the Value of E/W.

Time (min)	Oral temp.	Pulse rate	Blood pressure	Pulse pressure	Mean pressure	E/W
0	36.0	80	162/120	42	141	0.79
40	36.7	88	168/120	48	144	0.98
95	36.8	80	166/120	46	143	0.86
180	38.2	132	170/120	50	145	1.52
210	39.0	144	190/110	80	150	2.56

and Böger's estimate of the relationship $\frac{\Delta V}{V_s} = 0.5$ and will be proportionately altered if other estimates are used. E/W has the dimensions seconds^{-1}, and its value in the normal young adult will approximate unity.

The clinical values of ΔP and P_m in mm mercury may be used directly in the formula instead of the absolute values in dynes/cm^2, since E/W contains the ratio of the two pressures. Thus, in a subject with heart rate of 70, and arterial blood pressure of 110/70, E/W may be computed as follows:

$$r = 70,$$

$$\Delta P = 110 - 70 = 40,$$

$$P_m = \frac{110 + 70}{2} = 90,$$

$$E/W = \frac{r \cdot \Delta P}{30\, P_m} = \frac{70 \cdot 40}{30 \cdot 90} = 1.04.$$

The procedure described involves certain approximations and assumptions, the validity of which seems to be borne out by the observation that E/W, thus computed, in a variety of states varies in the direction which might be expected on the basis of generally accepted physiological concepts, as illustrated below.

[3] Grollman, A., *The Cardiac Output of Man in Health and Disease*, 1932, 179.

1. *The Effects of Epinephrine.* (Table I.)

The data included measurements of cardiac output, which showed a substantial increase after the injection of epinephrine. Grollman, in commenting on the data, said: "The increased cardiac output with the maintenance of a constant mean blood pressure must obviously indicate a decrease in the peripheral resistance. Hence despite its local vasoconstricting effect on arterioles epinephrine apparently causes vasodilatation when administered subcutaneously in man." The rise in the value of E/W fully supports Grollman's inferential reasoning.

2. *The Effects of Pitressin.* (Table II.)

The sharp drop in the value of E/W is consonant with the known vasoconstricting properties of pitressin, *i.e.*, the peripheral resistance is increased.

3. *The Effects of Pyrotherapy.* (Table III.)

The subject was a white woman *aet.* 34, suffering from benign hypertension of more than two years duration. There were minimal fundus changes and negative urine findings.

The marked increase in the value of E/W parallels the vasodilatation caused by the heat therapy, with the resultant decrease in peripheral resistance.

TABLE IV.†
The Change in the Value of E/W with Time Following a Period of Maximal Muscular Exercise.

Time after exercise (min)	Pulse rate	Blood pressure	Pulse pressure	Mean pressure	E/W
0.75	140	151/60	91	105	4.04
1.0	124	149/58	91	103	3.64
1.75	123	150/59	91	104	3.58
2.5	100	152/55	97	103	3.14
3.25	91	141/64	77	102	2.29
4.5	80	130/62	68	96	1.89
6.0	82	122/68	54	95	1.55
10.3	83	121/71	50	96	1.44
13.0	74	119/73	46	96	1.18
16.0	78	119/73	46	96	1.25
22.75	80	114/74	40	94	1.13
31.0	75	118/78	40	98	1.12
44.75	72	115/74	41	94	1.05
54.75	74	117/75	42	96	1.08

4. *The Effects of Muscular Exercise.* (Table IV.)

The subject, a normal young male, climbed 19 meters of stairs as rapidly as possible (about 25 seconds).

The high value of E/W immediately after exercise reveals the extremely low peripheral resistance, and hence the high degree of peripheral vasodilatation (confirming Ogden and Shock†).

The foregoing cases have all been analyzed on the implicit assumption that the value of E was not materially changed. It is clear that the value of E/W will be dependent on the values of both E and W. The exact analysis of cardiovascular dynamics requires determination of the absolute values of E and W, but in many cases, as in the above, it may reasonably be assumed that one of the variables is relatively stable compared with the other.

Summary. 1. A method of computing the ratio of aortic rigidity (or capacity) to peripheral resistance is described, using clinical values of heart rate and blood pressure. 2. The assumptions implicit in the method are discussed. 3. Several types of cardiovascular events are analyzed in terms of this ratio.

† Compiled from data collected by one of us in connection with the Adolescent Study of the Institute of Child Welfare, University of California. The facilities afforded are acknowledged with thanks. PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 5.

14229 P

Localized Heat Production in Living Tissue at a Distance.*

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The observations here reported were designed to test a method for injuring or destroying specific, localized areas of living tissue at a distance. The underlying principle of the method was the known heating effect upon metal of an electromagnetic field. The method consisted of implanting in the target areas of cats and rats small pieces of metal and then placing the whole animal in an electromagnetic field.

Source of the Electromagnetic Field. The source used for developing the electromagnetic field consisted of a quenched gap high frequency generator designed to operate from a 60 cycle power supply, requiring several kilowatts when operating at maximum output.

The field on the high frequency or secondary side of the generator was built up by means of a water cooled inductance, wound on a tube of bakelite of a size sufficient to accommodate the experimental animal and to prevent direct contact with any part of the winding during treatment.

In those experiments reported here, the frequency of the field was fixed at 400,000 cycles per second, relatively low compared with the frequencies used in diathermy. The secondary current, or that creating the field, reached values as high as 122 amperes.

Tests With Various Metals. After some preliminary experiments had demonstrated the feasibility of the method, tests of 3 metals were carried out in order to discover their relative suitabilities for this purpose. It was found that magnetic iron was better than cold rolled steel and that both were very much more useful than silver.

The difference between metallic iron and cold rolled steel is illustrated by their relative speeds in melting paraffin under the conditions of the experiment. Plates of metal, 3 x 3 mm were imbedded in paraffin with a melting point of 55°C, so as to be surrounded by about 3 mm of paraffin on all sides. The blocks of metal-containing paraffin were placed in a coil with an internal diameter of 4 inches. Sixty amperes of current were delivered to the coil. The relative lengths of time required to melt the paraffin are shown in Table I.

TABLE I.

Metal		Melting time, sec
Magnetic iron	.001 thickness	28
	.002 "	22
Cold rolled steel	.001 "	45
	.002 "	30

With the cold rolled steel plates, a temperature level of 113°F was reached in 30 seconds. The temperatures were measured with a specially made alcohol (not mercury) thermometer.

Tests With Animals. For experiments on the brains of rats, a coil 2.5 inches in internal diameter was employed. The rats were anesthetized with nembutal. The skull was opened, and a piece of magnetic iron wire 5 mm in length and 1 mm in diameter was inserted into the hemisphere at right angles to the surface and so that no wire was visible at the surface. The skull and soft tissues were then closed. In one experiment 90 amperes and in another 100 amperes were delivered for

* This work was done in connection with other studies supported by the Diamond Jubilee Fund and the Louis Wiley Memorial Fund. The latter was contributed to by Messrs. Ely M. Aaron, A. Beller, Walter Case, Mr. and Mrs. Edwin L. Coyle, Mrs. G. Richard Davis, Messrs. Leopold Demuth, Louis Golde, Edwin Goodman, Arthur Lamport, James Marshall, Max Meyer, Edward A. Norman, Gilbert Rhodes, Mrs. Jessie Waller and Mr. Louis S. Weiss.

We acknowledge with thanks the assistance of Samuel Lowis and Judah Ebin.

20 minutes. In both cases, a core of tissue 4 mm in diameter was destroyed (Fig. 1).

Extremely high or moderately high temperatures could be reached with varying speeds, depending upon the size of the coil, the amount of current delivered to it and the size and type of the metal. One cc of a water suspension of granulated iron, inserted by rectum into the colon of an ether-anesthetized rat with a syringe, for example, burned and destroyed the colon and adjacent tissues in 1 minute.

Temperatures were measured in a sampling manner only.

These observations are reported in order to exemplify a method which could have a

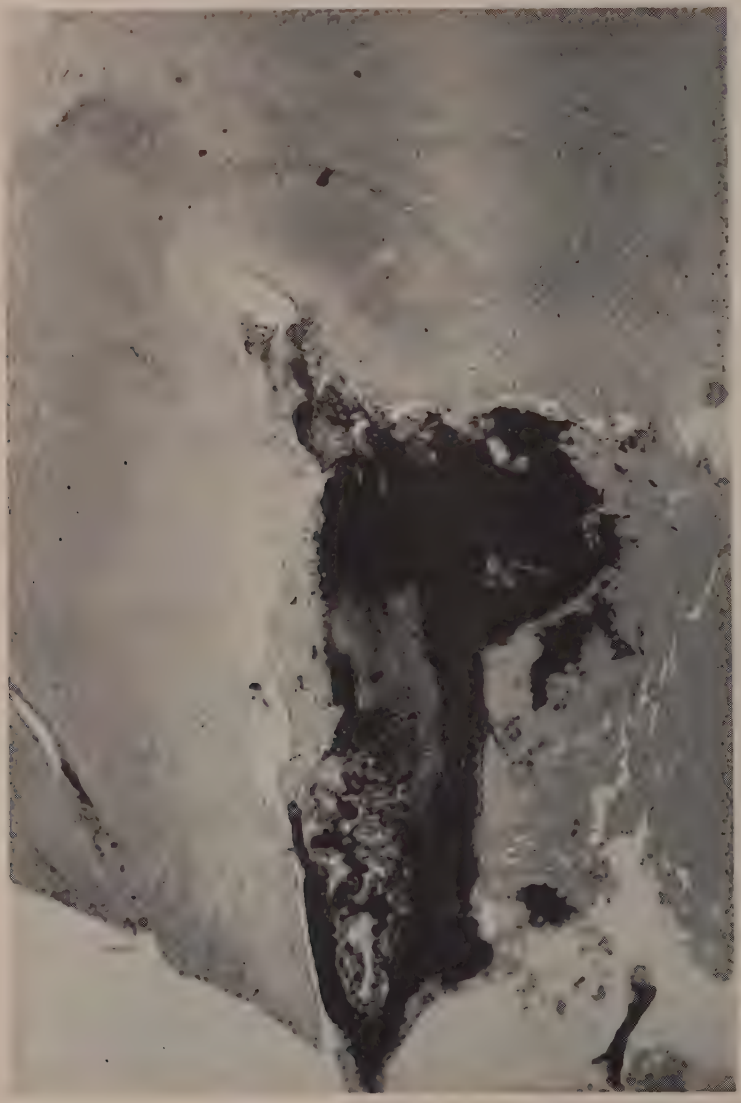

FIG. 1.
The burned area, bounded by a zone of less severely injured tissue. H. and E. stain.

variety of possible uses in experimental surgery, and perhaps even in clinical surgery. It might be especially applicable to areas difficult to approach, but which can be reached by a needle of wide calibre, through which wire, or granulated or perhaps powdered metal could be introduced.

14230 P

Influence of Spinal and Regional Anesthesia upon Vasoconstriction and Vasodilatation of Small Peripheral Blood Vessels.*

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In the current studies of the development of shock during surgical procedures, alterations in the rhythmical variations in the caliber of the small peripheral blood vessels of the fingers and toes have been noticed after spinal anesthesia and anesthetization of the stellate ganglion.

It is well known that spinal anesthesia may occasion a fall in blood pressure especially in the presence of moderate hemorrhage or trauma.[1,2,3,4] It is also known that peripheral blood vessels dilate when denervated of their sympathetic supply.[5] These two phenomena, fall in pressure and dilatation of the vessels, are related. What is still unknown is how those other small peripheral blood vessels with their sympathetic supply still intact participate in this result. If the blood pressure and the pulse rate were to remain constant, then, on the assumption that the cardiac output also remains constant, it may be assumed that, as one large set of vessels dilates, another contracts.

Method. The pneumoplethysmograph of Turner,[6] as modified by Neumann[7] was used to record the variations in volume of fingers and toes simultaneously. Normally, waves are inscribed representing constantly occurring changes in volume. These include the *pulse waves,* synchronous with the cardiac beat, and *alpha waves*[8] which occur 5 to 7 times per minute and vary in size up to 10 times that of the pulse waves.

Twelve patients free from hypertension and from peripheral vascular diseases, ranging in age from 24 to 54 years were studied. In 9, records were made during and after intrathecal injection of procaine hydrochloride or monocaine formate (150 mg). Sensory anesthesia as determined by testing sensory dermatomes reached the level of the umbilicus (T 12). In 3 patients, records were obtained before, during and after the injection into one stellate ganglion of 10 to 15 cc of 1½% solution of procaine hydrochloride. The clinical

* This is the 12th paper reporting the results of studies of the small blood vessels and related subjects.

The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

† Fellow of the University of Cincinnati, working on a grant from the Josiah Macy, Jr., Foundation.

1 Bradshaw, H. H., *Ann. Surg.*, 1936, **104**, 41.

2 Co Tui, F., *Arch. Surg.*, 1936, **33**, 825.

3 Seevers, M. H., and Waters, R. M., *J. A. M. A.*, 1932, **99**, 961.

4 Smith, H. W., Rovenstine, E. A., Goldring, W., Chasis, H., and Ranges, H. A., *J. Clin. Inv.*, 1939, **18**, 319.

5 Johnson, C. A., *Surg. Gynec. and Obstet.*, 1940, **70**, 31.

6 Turner, R. H., *J. Clin. Inv.*, 1937, **16**, 777.

7 Neumann, C., *Am. J. Physiol.*, 1943, **138**, 618.

8 Burch, G. E., Cohn, A. E., and Neumann, C., *Am. J. Physiol.*, 1942, **136**, 433.

signs regularly observed after successful injection of the stellate ganglion were uniformly present.

The subjects were recumbent and the fingers or toes were within 6 inches of the level of the heart. None of the patients knew when the anesthetic agent was injected. Records were obtained in the absence of surgical trauma or hemorrhage.

Observations. Before anesthesia, the needles being in place, the tracings were normal, the pulse waves being 6 to 7 cu mm‡ and the alpha waves up to 60 cu mm. Two minutes afterward, in the case of spinal anesthesia, the pulse waves in the toe became progressively larger (up to 15 cu mm) while the alpha waves became smaller and almost disappeared. Concurrently, in the fingers, the size of the pulse waves progressively decreased to one-half or even one-quarter their former size, while the size of the alpha waves decreased, as in the toes.

Similar effects were noticed on injection of one stellate ganglion. Pulse waves of the ipsilateral fingers increased and alpha waves decreased, while in the opposite fingers both waves, pulse and alpha, decreased to one-half or one-quarter the original size. There were no marked alterations in blood pressure or pulse rate during the period of spinal or regional anesthesia.

Such striking changes were not observed when occlusive vascular diseases were present (3 cases). Cardiac output and volume of visceral flow were not measured.

Comments. These observations indicate that when marked dilatation occurs in one peripheral vascular bed, owing to paralysis of the sympathetic supply, concomitant vasoconstriction occurs in certain other remote peripheral beds. The mechanism involved seems to be one of adjustment, possibly compensation, through efferent pathways of the sympathetic system. Paralysis of these nerves permits maximal expansion of the small blood vessels (large pulse waves) and interrupts the pathway of stimuli necessary for the formation of prominent alpha waves; at the same time, increased sympathetic outflow to certain other vascular fields results in vasoconstriction intense and persistent enough to produce small pulse waves continuously and to inhibit the relaxation apparently necessary for the development of alpha waves. It seems that such alterations in the patterns of alpha and pulse waves reflect changes in the activity of certain sympathetic pathways. The method may be useful, then, for clinical studies of the effects of numerous physiological stresses, such as those preceding shock, upon such pathways and upon small peripheral blood vessels.

‡ All changes in volume are calculated for finger tips or toe tips of 5 cc.

14231 P

Tubular Resorption of Chloride in Essential Arterial Hypertension: Intensive Study of One Case.*

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In a previous study[1] we have compared a group of individuals with essential hypertension and a control group of normal subjects, with respect to tubular resorption of chlorides. In the present study we have taken 2 male subjects of comparable age and investigated them extensively by the same technique, namely chloride excretion considered in terms of the urine concentration of inulin divided by the plasma concentration, or the U/P inulin ratio.

Methods. The experimental subject was a retired business man, 55 years of age, who

* Funds for this study were granted through the Alice Cook Guild Memorial Fund, established by Kenneth G. Smith.

1 Farnsworth, E. B., and Barker, M. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 74.

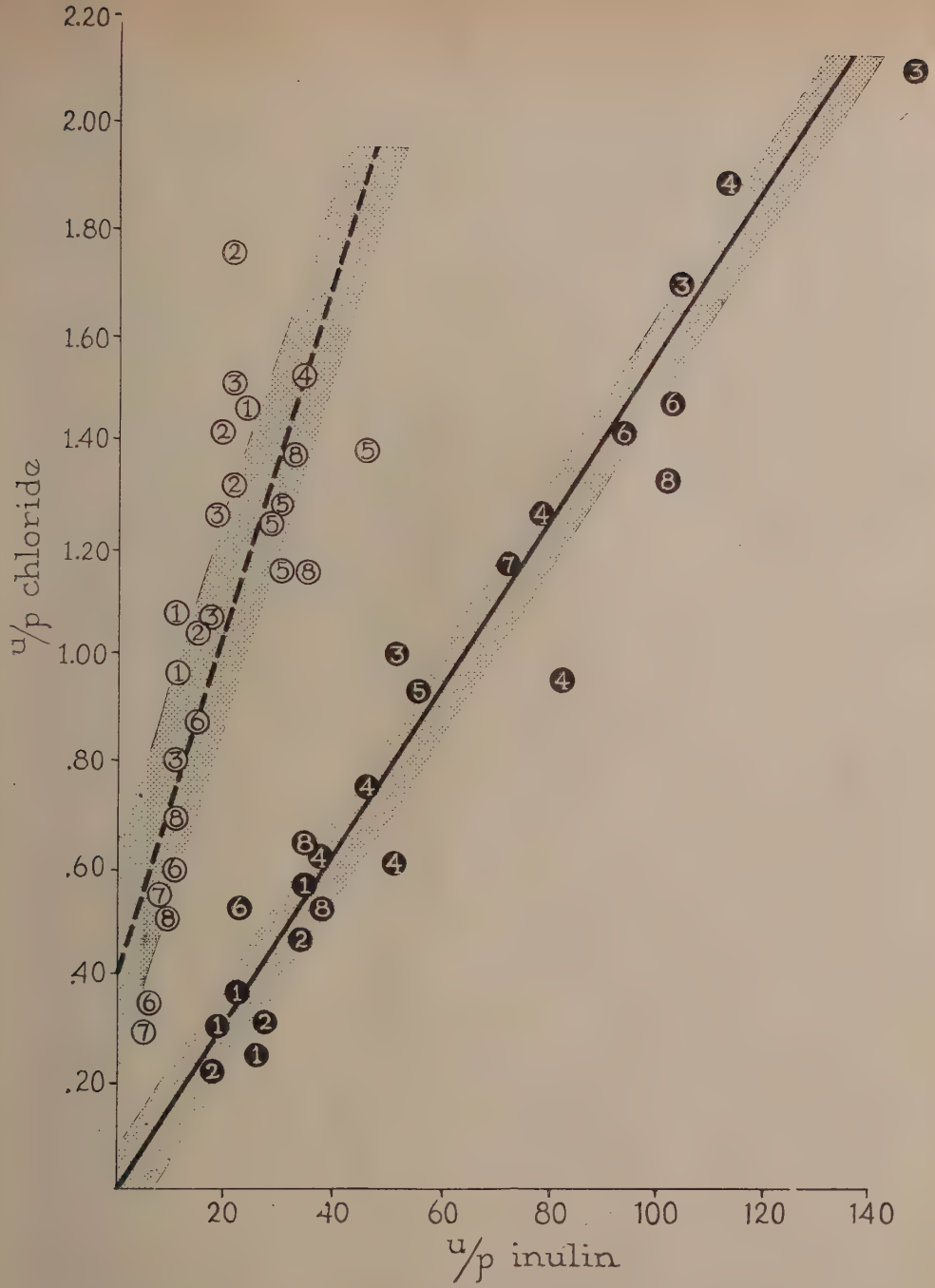

FIG. 1.

Black symbols represent the normal subject; white symbols represent the patient with essential arterial hypertension. Symbols containing the same number indicate successive clearance period performed on the same day.

The coefficient of linear correlation for the normal subject is .989 ± .0045 with a standard error of estimate of .09.

The coefficient of linear correlation for the hypertensive patient is .894 ± .0261 with a standard error of estimate of .18.

gave a history of hypertension of long standing. His complaints were of failing vision, disturbed memory and headaches. Urine analysis showed no albumin, sugar or cells. The erythrocyte count was 4,850,000, the hemoglobin 13.0 g. He was without evidence of edema. Ophthalmoscopic examination of the eye-grounds showed diffuse angiospasm, without retinopathy. The blood urea nitrogen, non-protein nitrogen, cholesterol and proteins were within normal limits. The systolic blood pressure ranged from 200 to 234, the diastolic from 106 to 134.

The normal subject was a 53-year old railroad employee who was hospitalized because of a fracture. He had no complaints and gave no evidence of other pathology. His personal and family history was negative for cardiovascular or renal disease. The urine was without albumin, sugar or cells. The erythrocyte count was 5,100,000, the hemoglobin 15.0 g. The blood pressure was 140/84.

These 2 individuals were studied by a method consisting of concomitant clearances of inulin, diodrast, and chloride. The U/P chloride was plotted against the U/P inulin. Both subjects were on the standard ward diet and received similar preparation. As in the previous study, the blood was taken under oil, and estimations were made on serum and quoted in terms of sodium chloride. Analysis of chloride concentration was carried out by the method of Sendroy.[2] Determinations on inulin were made by the method of Corcoran and Page.[3] Clearance periods ranged from 15 to 30 minutes in length. The normal subject is represented by a total of 24 periods, the hypertensive subject by 27.

Results. Multiple tests of these 2 individuals corroborated our experience in the group study previously undertaken. The hypertensive subject was found to reabsorb substantially less chloride than did the normal subject.

Discussion. The significance of this deviation of the hypertensive kidney from the normal is far from clear. In order to determine whether or not the phenomenon was specific to chloride, we ran simultaneous tests on phosphate and subjected them to similar analysis. It was found that phosphate excretion in the hypertensive subject showed only an insignificant and irregular increase over the normal. This fact seems to suggest that chloride resorption is affected by certain factors which do not govern the fate of other metabolites similarly filtered.[4]

The average clearances of the 2 subjects are given in the accompanying table.

TABLE I.

	Hypertension (C.V.)	Normal (C.P.)
Inulin	58 cc/min	79 cc/min
Diodrast	249	430
Chloride	3.3	1.2
Phosphate	16.3	12.3

Conclusions. Repeated clearance periods taken on an individual with essential arterial hypertension and compared with those taken on a normal subject demonstrated that there was substantially less reabsorption of chloride in the hypertensive individual. This diminished resorption of chloride in the tubules of the hypertensive subject, at all rates of water resorption, is offered as evidence suggestive of a specific modification of chloride treatment in essential arterial hypertension.

2 Sendroy, J., *J. Biol. Chem.*, 1937, **120**, 2.

3 Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 601.

4 Walker, A. M., *J. Biol. Chem.*, 1933, **101**, 239.

14232

Effect of Striated Muscle Paralysis Induced with Erythroidine upon the Electroencephalogram (E. E. G.)

EDWARD GIRDEN.*

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In a series of previous studies, an analysis was made of the psychosomatic relations during the development of conditioned reflexes in curarized and erythroidinized mammals.[1-7] It was shown that there was a central as well as a peripheral effect, the electrical and extirpation data[1,2,3] indicating that the former consists of a depression of synaptic transmission in the cerebral cortex. A recent investigation by Feitelberg and Pick suggests that the cortical cell metabolism, as measured by the electroencephalogram, also suffers functional inactivity since the E. E. G. is completely abolished in the curarized frog.[8] The present study is concerned with a test of the same question in mammals.

The E. E. G. was recorded in a shielded room on an ink-writer activated by a single high-gain channel amplifier (approximate range was 1-40 cycles, sensitivity that of 0.4 mm/mv). Operative preliminaries, implanting of electrode (when pickup was desired in the normal before drug-injection), exposure of cortex, cannulation (in monkeys), all were performed under ether anesthesia. Breathing in the drugged animal was maintained by a Harvard respirator. The drug consisted of a 1% or 2% water-solution of dihydro *B* erythroidine hydrobromide.

Dosage for 10 dogs (intravenous injection) is presented in Table I. Incidental observations obtained from several other dogs, conforming with the data reported below, are excluded only because they lacked completeness. Two *Macacus rhesus* monkeys (1.0 and 1.5 kilos respectively), each subjected to a total dosage of 125 mg (intraperitoneal injection), were also studied. A great number of tests were made with visual and acoustic stimuli and in relation to the nature of the pickup from different cortical areas. In general, in an animal weighing from 2 to 4 kilos, complete recovery would not occur until 1 to 1½ hours after a single injection of 20 to 60 mg erythroidine, and a single dose of 100 mg

TABLE I.
Dosage of Erythroidine.

(I) Animal No.	(II) Wt in kilos	(III) Initial doses	(IV) Total dosage	(V) Duration in min
R-51	4.0	20, 70	290 (2)	140
R-52	9.0	10, 30	250 (2)	100
R-54	9.0	20, 20	140 (1)	132
R-55	7.0	50	350 (3)	68
R-56	2.0	10, 30, 60	100	32
R-57	2.0	60	160 (1)	40
R-58	4.0	60	60	84
R-59	2.0	10, 20, 10	100	95
R-60	3.2	5, 30	355 (3)	163
R-61	2.5	60, 80	240 (1)	52

Dosage is given in mg; in (III) the initial injections are given separately, in (IV) the total dosage is given and the number in parenthesis indicates the number of single injections each of 100 mg. Thus in dog R-51, the first two spaced doses were 20 mg and 70 mg respectively; later 2 subsequent injections of 100 mg each were made (total dosage 290 mg). The duration of the experiment (V) was counted from the time of the first injection until the animal was dispatched.

* Fellow of the John Simon Guggenheim Memorial Foundation, 1941-42. Special aid from the Penrose Fund of the American Philosophical Society is gratefully acknowledged. The erythroidine was generously supplied by Merck & Company, Rahway, New Jersey.

† Communication No. 51 from the Physiological Psychology Laboratory, University of Rochester, maintained by aid of the Research Council, American Otological Society.

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2 Culler, E., Coakley, J. D., Shurrager, P. S., and Ades, H. W., *Am. J. Psychol.*, 1939, **52**, 266.

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8 Feitelberg, S., and Pick, E. P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 654.

would insure complete paralysis from 2 to 4 hours. As shown in Table I, the total dosage in many cases far exceeded the latter value. Greater reliance was obtained with functional criteria, and all stages were tested, from no observable change to complete striated muscle paralysis.

In general the E. E. G. in the drugged animal conformed to that recorded in the normal condition. There was some evidence that the magnitude of the pick-up in certain cases was reduced after the animal was erythroidinized, but the wave-form remained undisturbed. A series of supplementary doses, introduced subsequently, each one sufficient to produce complete muscular paralysis, likewise failed to disturb the E. E. G. On occasion, the E. E. G. was permanently distorted following the injection of a single massive dose (sufficient to produce complete paralysis for more than 4 to 6 hours). This effect was probably due to disturbed metabolism (reduction in the amount of available oxygen) since the blood pressure falls, temporarily, following the injection of even moderate dosage of the drug.[5,7] When artificial respiration was eliminated, the E. E. G. disappeared, to reappear undistorted, or permanently impaired, depending upon the length of the unrespirated interval. This effect, however, was not due to the direct action of the drug upon the cortex, but rather the indirect consequence of the lack of oxygen required to sustain normal cortical metabolism. Unlike the mammal, the curarized frog survives without tracheal insufflation due to the presence of the accessory skin respiratory mechanism. The disappearance of the E. E. G. in the curarized frog therefore, is more likely produced by the reduction in available oxygen rather than the *direct* effect of curare upon the cortex, as maintained by Feitelberg and Pick.[8]

Libet et al. have also reported the persistence of the cortical activity in curarized dogs subjected to metrazol.[9] No extended tests were made by these investigators with curare alone, and the metrazol disturbed the E. E. G. considerably. Their results, however, are in agreement with the present data in that the E. E. G. did not disappear following curarization, or, for that matter, after the metrazol took effect.

Summary. So long as proper artificial respiration is given, normal cortical activity, as measured by the E. E. G., persists undisturbed in both the dog and the monkey during complete striated muscular paralysis induced with erythroidine. The difference in results reported here with mammals and that previously reported by other workers in the frog (complete elimination of the E. E. G.) is interpreted to be due to a reduction in the available oxygen necessary for normal cortical metabolism in the latter organism.

[9] Libet, B., Fazekas, J. F., and Himwich, H. E., *Am. J. Psychiat.*, 1940, **97**, 366.

14233 P

Some New Choline Esters with Cycloplegic and Mydriatic Action.*

KENNETH C. SWAN AND NORMAN G. WHITE. (Introduced by E. G. Gross.)

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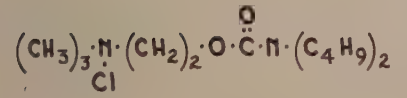
Choline derivatives such as acetylcholine and carbamylcholine (doryl) produce constriction of the pupil and contraction of the ciliary muscle. The halide salts of these compounds have little effect on the surface tension of water, *i.e.*, they are relatively surface-inactive; as surface activity has a profound influence on many biologic phenomena, it was believed choline derivatives which were surface-active might have an enhanced or even

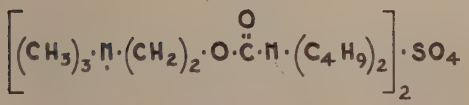
* Part of a study being conducted under a grant from the John and Mary R. Markle Foundation.

TABLE I

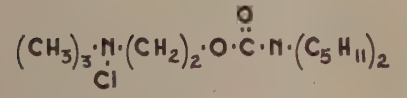
Surface-active Carbamylcholine Derivatives



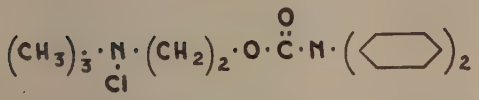
Di-n-butyl-carbamylcholine chloride
and
Di-iso-butyl-carbamylcholine chloride



Di-n-butyl-carbamylcholine sulphate



Di-n-amyl-carbamylcholine chloride



Di-phenyl-carbamylcholine chloride

different pharmacological effect. Investigation of this possibility necessitated synthesis of new compounds in which highly hydrophilic choline salts were combined by an ester linkage with highly hydrophobic groups. The resultant elongated molecules would be predominantly hydrophilic at the choline end and predominantly hydrophobic at the opposite end. Molecules with this structure would be surface-active.

The first series of surface-active choline esters have been synthesized (Table 1). The $-NH_2$ radical of carbamylcholine was replaced by water insoluble amines. Five to 10% solutions of the chloride salts of these drugs have a water-air interfacial tension of less than 50 dynes per cm at 25°C. In the albino rabbit, mydriasis develops promptly after a single instillation of each of these solutions into the conjunctival sac. The mydriasis results from paresis of the iris sphincter for the drugs produce no pupillary changes in eyes in which the iris sphincter has been removed surgically. In the human eye, mydriasis is accompanied by cycloplegia. The action of the new drugs, therefore, resembles that of atropine and is opposite to that of carbamylcholine and acetylcholine.

Substitution of ethyl for the methyl radicals of the quaternary ammonium group increases the mydriatic action of these new drugs, but decreases the cycloplegic properties. Replacement of the methyl by propyl and butyl radicals reduces the intensity of both mydriatic and cycloplegic effects.

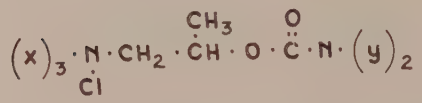
Branched chain compounds, *e.g.*, di-isobutyl carbamylcholine, are pharmacologically less active than the corresponding straight chain isomers.

The members of the beta-methyl carbamylcholine series (Table II) have the same qualitative effects on the eye as the corresponding carbamylcholine compounds.

The aromatic derivatives, *e.g.*, diphenylcarbamylcholine chloride, have less mydriatic and cycloplegic properties than corresponding

TABLE II

β-methyl-carbamylcholine Derivatives



x = Methyl, Ethyl, Propyl radicals
y = Butyl, Amyl, Phenyl radicals

di-n-butyl compounds, particularly in the human eye.

Instillations of 7.5% aqueous solutions of di-n-butyl carbamylcholine chloride were made into the conjunctival sacs of rabbits several times daily for 4 weeks. No evidence of injury to the intraocular tissues was noted by either histologic methods or by ophthalmoscopy and slit lamp biomicroscopy. A transient, superficial, punctate disturbance of the corneal epithelium frequently followed instillations of more concentrated solutions. A similar disturbance was noted in the human eye.

Systemic pharmacology and toxicology of the new compounds are to be studied by one of us† in partial fulfillment of requirements for a doctorate degree, and will be reported separately; however, no systemic effects have been noted following instillations into the conjunctival sac of adult patients.

The only clinical application of the new drugs has been in routine intraocular examination and cycloplegic refraction. For this purpose, di-n-butyl-carbamylcholine sulfate has been the most satisfactory. Two instillations of a 7.5% solution into the conjunctival sac produce mydriasis and cycloplegia beginning within 20 minutes and becoming maximal in 60 to 90 minutes. The reactions of the intraocular muscles usually return to normal 7 to 12 hours after administration. The duration of action is, therefore, relatively short. In this respect, di-n-butyl-carbamylcholine resembles homatropine rather than atropine.

A comparative study of di-n-butyl-carbamylcholine sulfate and homatropine was made on 400 patients. Each patient received 2 instillations of 5% homatropine hydrobromide in one eye and 2 instillations of a 7.5% solution of the new drug in the other. There was little difference in the degree of mydriasis and cycloplegia produced by the two drugs; however, cycloplegia and particularly mydriasis was less prolonged following di-n-butyl-carbamylcholine.

Only the most promising members of the new class of drugs have been investigated. Synthesis and pharmacologic investigation of surface-active choline esters other than the carbamate series are being continued.

Summary. A new class of choline esters with mydriatic and cycloplegic properties is reported. For routine examinations, di-n-butyl-carbamylcholine sulfate produces cycloplegia and mydriasis comparable in degree to that produced by homatropine; however, the new compound has the advantage of producing a shorter period of visual disability.

† N. G. White.

14234

Heredity of Variants of the Rh Type.*

A. S. Wiener and Karl Landsteiner.

From the Office of the Chief Medical Examiner and the Rockefeller Institute for Medical Research of New York City.

By immunizing animals with the blood of rhesus monkeys, Landsteiner and Wiener[1] obtained antisera which agglutinated approximately 85% of human bloods from white individuals in New York City, independently of the blood groups and other known agglutinogens. The agglutinable property in human blood responsible for the reaction was designated as Rh. Subsequently sera giving parallel reactions were obtained from patients who had had hemolytic reactions to blood transfusions of a homologous group[2] and from mothers of erythroblastotic babies.[3] Among the human sera two varieties were found, one of which, the more common, gave about 3% more positive reactions.[4]

Furthermore, some human anti-Rh sera were encountered which agglutinated only about 70% of human bloods,[5] that is, only about five-sixths of the Rh positive bloods, so that a major subdivision within the Rh positive type was demonstrated. Human sera of this type are rather uncommon.

In place of the nomenclature suggested by one of the writers,[6] the following less complicated designations are now suggested (cf.

TABLE I.
Classification of the Subtypes of Rh.

Anti-Rh serum	Designation of agglutinin in serum	Approximate % positive reactions	Reactions of various bloods among white individuals (New York City)			
			About 70%	About 3%	About 14%	About 13%
1.*	Anti-Rh	84	Pos.	Neg.	Pos.	Neg.
2.†	Anti-Rh_1	73	,,	Pos.	Neg.	,,
3.‡	Anti-Rh′	87	,,	,,	Pos.	,,
	Designation of types		Rh positive			Rh negative
	Designation of main subtypes			Rh_1	Rh_2	
				Rh′		

* Landsteiner, K., and Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1940, **42**, 223.

† Wiener, A. S., *Arch. Path.*, 1941, **32**, 227; Landsteiner, K., and Wiener, A. S., *J. Exp. Med.*, 1941, **74**, 309.

‡ Wiener, A. S., *Arch. Path.*, 1941, **32**, 227; Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

* One of the authors (A. S. W.) was aided by a grant from the Carnegie Corporation and the Committee on Human Heredity of the National Research Council.

1 Landsteiner, K., and Wiener, A. S., Proc. Soc. Exp. Biol. and Med., 1940, **42**, 223.

2 Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

3 Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

4 The immune anti-rhesus sera from guinea pigs correspond to the human antisera giving about 84% positive reactions with bloods from N. Y. white individuals, as will be shown in a separate publication (Landsteiner, K., and Wiener, A. S., in preparation). However, it may be noted that these human sera are apparently not entirely identical in their reactions.

5 Wiener, A. S., *Arch. Path.*, 1941, **32**, 227; Landsteiner, K., and Wiener, A. S., *J. Exp. Med.*, 1941, **74**, 309.

6 Wiener, A. S., *Blood Groups and Transfusion*, p. 254, C. C. Thomas, Springfield, Ill., 1943.

Table 1). When a distinction between the various subtypes of Rh is desired, the great majority of bloods, namely, those which react also with serum No. 2 of the table, are designated as Rh_1. Of these, a small percentage fail to react with guinea-pig antisera or human sera listed as No. 1 and are designated as Rh.' Those bloods which do not react with sera anti-Rh_1 (No. 2) are called Rh_2. The agglutinins are named so as to correspond to agglutinogens of the blood cells. The new nomenclature described above is suggested with the approval of Dr. Philip Levine.

Bloods of subtype Rh_2 may give weaker re-

TABLE II.
List of Family Material.

Family No.*	Parents: Father	Parents: Mother	Children				
1	$OMNRh_1$	$OMNRh_1$	$OMRh_1$ ♀	$OMRh_1$ ♂	$OMNRh_1$ ♂	$OMRh_1$ ♀	
2	$OMNRh_1$	$OMNRh_1$	$ONRh_1$ ♂	$OMNRh_1$ ♂	$OMNRh_1$ ♂		
3	$OMNRh_1$	$BMNRh-$	$BMNRh_2$ ♂	$ONRh_1$ ♂	$BMNRh_2$ ♂	$BNRh_2$ ♂	
4	$OMRh_1$	$BNRh_1$	$BMNRh_2$ ♂	$OMNRh_2$ ♂	$OMNRh_1$ ♀	$OMNRh_2$ ♀	
5	$OMNRh_1$	A_1NRh_1	$ONRh_1$ ♂	$OMNRh_1$ ♀	$OMNRh_1$ ♂		
6	$BMRh_1$	$OMNRh_1$	$OMRh_1$ ♀	$BMNRh_1$ ♀	$OMNRh_1$ ♀	$BMRh_1$ ♂	
7	A_2MNRh_1	$BMRh_1$	A_2MRh_1 ♀	$BMRh_1$ ♀			
8	A_1MNRh-	$OMNRh_1$	A_1MRh_1 ♀				
9	A_2MNRh-	A_2MRh_2	A_2MRh_2 ♀	A_2MRh_2 ♂	A_2MRh_2 ♀	A_2MNRh_2 ♀	
10	A_1MRh_1	A_1MRh_2	A_1MRh_1 ♀	A_1MRh- ♀			
11	A_1MRh_2	$OMRh_1$	$OMRh_2$ ♂	A_1MRh_1 ♂	A_1MRh_2		
12	A_1MNRh_1	$ONRh-$	A_1MNRh- ♂	A_2MNRh_1			
13	A_1NRh_1	A_1MNRh_2	A_1MRh_2 ♀ † A_1MNRh_1 ♂	A_1MNRh_1 ♀	A_1NRh_1 ♀	A_1MNRh_1 ♂	A_1NRh_2 ♀
14	$OMRh-$	$OMRh_1$	$OMRh_1$ ♂	$OMRh_1$ ♂			
15	$OMRh_1$	$OMNRh_1$	$OMRh_1$ ♀	$OMNRh_1$ ♀	$OMNRh_1$ ♀		
16	A_2MRh_2	$BNRh_1$	$OMNRh-$ ♀	A_2BMNRh_1 ♂	$BMNRh_1$ ♂		
17	$OMRh_1$	$OMNRh_2$	$OMNRh_1$ ♂	$OMRh_1$ ♂	$OMNRh_1$ ♀		
18	$OMNRh_1$	$OMRh_1$	$OMNRh_1$ ♀	$OMNRh_1$ ♂	$OMNRh_1$ ♂		
19	A_1MNRh_1	A_1NRh_1	A_1NRh_1 ♂				
20	$OMRh_1$	A_1MRh-	A_1MRh_2 ♀	A_1MRh_1 ♂	A_1MRh_2 ♀	A_1MRh_1 ♂	A_1MRh_2 ♀
			A_1MRh_1 ♂	A_1MRh_2 ♂	A_1MRh_1 ♀	A_1MRh_2 ♂	A_1MRh_1 ♂
21	$BMNRh_1$	A_1NRh_2	$OMNRh_1$ ♂	$BNRh_1$ ♀			
22	A_1MRh_1	A_1NRh_1	A_1MNRh_1 ♀	A_1MNRh_1 ♀	A_1MNRh_2 ♀	$OMNRh_1$ ♂	
23	A_1MNRh_2	$BMRh_1$	A_1MNRh_1 ♂	A_1BMNRh_1 ♂			
24	A_1MRh_1	A_1MRh_1	A_1MRh_1 ♀	A_1MRh_1 ♀			
25	$OMRh_1$	$OMNRh_1$	$OMNRh_1$ ♂	$OMRh_1$ ♀	$OMNRh_1$ ♀		
26	$OMRh_2$	$OMRh_2$	$OMRh_2$ ♂	$OMRh_2$ ♀			
27	A_1BNRh-	$ONRh-$	$BNRh-$ ♂	$BNRh-$ ♂			
28	A_2MRh_1	$OMNRh_1$	A_2MRh_1 ♂	A_2MNRh_1 ♀	$OMNRh-$ ♀		
29	A_1MRh_1	A_2MNRh_1	A_2MNRh- ♀	A_1MRh_1 ♂	$OMRh_1$ ♀		
30	$OMRh_1$	$ONRh-$	$OMNRh_1$ ♂	$OMNRh_1$ ♀			
31	$BNRh_1$	$OMNRh_1$	$OMNRh_1$ ♀	$OMNRh_1$ ♂	$ONRh-$ ♀	$ONRh_1$ ♂	$ONRh-$ ♀
32	A_1NRh_1	$OMRh_1$	$OMNRh_1$ ♀	$OMNRh_1$ ♀			
33	$OMNRh_1$	$OMRh_1$	$OMNRh-$ ♀	$OMRh_1$ ♀			
34	$OMNRh_1$	$BMNRh_1$	$BNRh_1$ ♂	$OMRh_1$ ♀			
35	$BNRh_1$	A_2MRh_1	$OMNRh_1$ ♀	A_2MNRh- ♂	$BMNRh_1$ ♀		
36	A_1MNRh_2	$OMNRh_1$	A_1MNRh_1 ♂	A_1NRh_1 ♂			
37	$BNRh_1$	$BMRh_1$	$BMNRh_1$ ♂	$BMNRh_1$ ♂	$BMNRh_1$ ♀	$OMNRh_1$ ♂	
38	$OMNRh_1$	A_1MNRh_1	A_1NRh_1 ♂	A_1MNRh_1 ♀			
39	A_1NRh_1	$OMNRh_1$	A_1MNRh_1 ♀	$OMNRh_1$ ♂	A_1MNRh_1 ♀		
40	A_2BNRh-	$OMNRh_1$	$BMNRh_2$ ♀				
41	$ONRh_1$	$OMNRh-$	$OMNRh_1$ ♂	$OMNRh_1$ ♂	$OMNRh_1$ ♀		
42	A_1MNRh_2	$ONRh_1$	A_1MNRh_1 ♂	A_1MNRh_1 ♂			
43	$BMRh_1$	A_1MNRh_1	$OMRh_1$ ♀	A_1BMRh_1 ♂			
44	A_1MNRh_1	$OMRh_1$	$OMRh_1$ ♂	A_1MRh_1 ♀			
45	A_1MRh_1	$OMRh-$	$OMRh_1$ ♂	A_1MRh_1 ♂			
46	A_1MNRh_2	$BMNRh_2$	A_2NRh- ♀	A_2BMRh_2 ♂			
47	A_1MRh_1	A_2MNRh_1	A_1MNRh_1 ♂	A_1MNRh_1 ♂			

* Families 1-7 are the same as families 10, 12, 24, 26, 31, 36, and 37 from the study of Landsteiner and Wiener.

† Child of former marriage.

TABLE III.
Heredity of Subtypes of Rh.

Mating	No. of families	Children of subtype			
		Rh_1	Rh_2	Neg.	Totals
$Rh_1 \times Rh_1$	25	61	4	6	71
$Rh_1 \times Rh_2$	9	19	4	2	25
$Rh_1 \times$ Neg.	9	17	9	1	27
$Rh_2 \times Rh_2$	2	0	3	1	4
$Rh_2 \times$ Neg.	1	0	4	0	4
Neg. $\times$ Neg.	1	0	0	2	2
Totals	47	97	24	12	133

actions than Rh_1 bloods with human antisera (anti-Rh'). Therefore, it may be advisable, when conducting tests with human anti-Rh sera, to include Rh positive controls of subtype Rh_2 as well as Rh_1.

We have studied the heredity of the major subdivisions of the Rh type in 47 families with 135 children (*cf.* Table II). It appears from Table III that the subtypes Rh_1 and Rh_2 resemble the subgroups of A in their hereditary transmission. Thus, it is postulated that there are 3 main allelic genes, Rh_1, Rh_2, and *rh*, where Rh_1 and Rh_2 are both dominant over *rh* and Rh_1 is dominant over Rh_2. This theory explains why no children of subtype Rh_1 were encountered in the 4 families with 10 children where neither of the parents belonged to subtype Rh_1. In this connection it may be mentioned that Levine[7] has found that in 17 families with 21 children in which the blood of neither parent was agglutinated by anti-Rh_1 serum, the bloods of all but one of the children failed to react with the serum, and in this single case the possibility of illegitimacy has to be considered.

A further test of the theory is provided by the matings Rh_1 x Rh_1 and Rh_1 x Rh negative. Since Rh_1 individuals must belong to genotype Rh_1Rh_1, Rh_1Rh_2, or Rh_1rh, these families, as is shown in Table II, fall into three groups, depending upon whether the children are all Rh_1, or Rh_1 and Rh_2, or Rh_1 and negative. From the theory it follows that when an Rh_1 parent in these matings has an Rh negative child, none of his children can belong to subtype Rh_2; on the other hand where the Rh_1 parent has given rise to an Rh_2 child, he cannot have Rh negative children. No exception to this rule was encountered in our series of families. A particularly striking case is family No. 20, in which the father is Rh_1 and the mother Rh negative. In this family there are 5 children of subtype Rh_1 and 5 of subtype Rh_2, fulfilling the theoretical expectation under the assumption that the father belongs to genotype Rh_1Rh_2.

Practical application of the heredity of the subtypes of Rh in cases of disputed parentage seems feasible and would afford a slight increase in the chances of exclusion. However, the difficulty in obtaining suitable reagents for the present prevents its general use. Moreover, much more extensive study of families would be necessary to place the theory on a basis safe enough for forensic purposes.

Based on the percentages of the types given in Table 1, the approximate frequencies of the genes are as follows:

$$rh = \sqrt{\text{Neg.}} = \sqrt{0.13} = 36\%$$
$$Rh_2 = \sqrt{\text{Neg.} + \text{Rh}_2} - \sqrt{\text{Neg.}} = \sqrt{0.27} - \sqrt{0.13} = 16\%$$
$$Rh_1 = 1 - (Rh_2 + rh) = 48\%$$

It may be noted that the reactions discussed do not exhaust the variety existing among human anti-Rh sera. Thus, recently a serum was encountered which gave strong agglutination with the great majority (but not all) of Rh_2 bloods and only a minority of Rh_1 bloods, while Rh negative bloods failed to react.[8] From a preliminary study, the agglutinogen responsible for the reaction appears to be inherited as a simple Mendelian dominant, probably due to a special allelic gene.

[7] Personal communication.

[8] The properties of this new anti-Rh serum will be presented in detail in a separate report.

Summary. Studies on 47 families with 133 children indicate that the major subtypes Rh_1 and Rh_2 are transmitted by means of corresponding allelic genes Rh_1 and Rh_2 which are both dominant over the gene *rh;* and, in addition, that gene Rh_1 is dominant over Rh_2. (A nomenclature for designating the subdivisions of the Rh positive type is proposed).

14235

The Tocopherol Level in Human Serum During Oral Tocopherol Therapy.*

ISRAEL S. WECHSLER, GERDA GERNSHEIM MAYER, AND HARRY SOBOTKA.

From the Neurological Service and the Department of Chemistry, Laboratories of the Mount Sinai Hospital, New York.

In a preceding publication[1] we have described the results of tocopherol studies in human serum by a photoelectric adaptation of the Emmerie and Engel α,α'-bipyridine method.[2] The average tocopherol level for a group of 12 healthy young individuals on an unrestricted diet was 0.96 mg/100 ml of serum. Patients suffering from amyotrophic lateral sclerosis showed slightly lower values within the normal range before tocopherol therapy. The blood level rose after oral administration of the vitamin, but in cases receiving solely intramuscular tocopherol injections a paradoxical drop was noted.

We have now increased the number of cases of amyotrophic lateral sclerosis† and added a group of patients treated orally with tocopherol for myopathies and miscellaneous diseases other than amyotrophic lateral sclerosis.‡ dl-α-Tocopherol acetate in 25 mg tablets was used in all experiments.

The average blood level for 17 untreated cases of amyotrophic lateral sclerosis was 0.67 mg/100 ml and that for 14 miscellaneous myopathies was 0.61 mg/100 ml before treatment. The figures in Tables I and II corroborate the observation that tocopherol ingestion, repeated for several successive days, raises the serum level significantly.

The patients who received treatment are

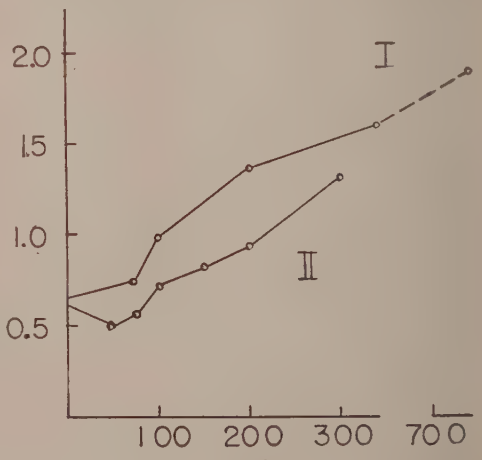


FIG. 1.

Average Serum Tocopherol Level after Administration of Ephynal to Patients with Amyotrophic Lateral Sclerosis (I) and Other Diseases (II).

Ordinates are mg Tocopherol/100 ml serum. Abscissæ are mg Tocopherol administered per day. The points in the graphs represent the average serum tocopherol level for each dosage group. The point given for "0 mg dosage" is the average of all pre-treatment determinations. If the post-treatment level for each dosage group were compared with the pre-treatment level of the corresponding individual group, insignificant changes in the rise of each group would result.

* This work was carried out under grants from the John and Mary Markle Foundation, New York, and the Hoffman La Roche Company, Nutley, New Jersey, who also supplied the Ephynal. We hereby gratefully acknowledge their generous aid.

1 Wechsler, I. S., Gernsheim Mayer, G., and Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 152.

2 Gernsheim Mayer, G., and Sobotka, H., *J. Biol. Chem.*, 1942, **143**, 695.

† A clinical study by I. S. Wechsler, M. R. Sapirstein, and A. Stein of 81 cases, including those studied here, will appear shortly.

‡ We are indebted to the Neurological Service of the Montefiore Hospital for permission to study cases No. 207, 211-213, included in this group.

TABLE I.
Rise in Serum Tocopherol in Amyotrophic Lateral Sclerosis Patients upon Oral Administration.

No.	Sex	Age	Tocopherol content of 100 ml serum: Before treatment mg	After treatment mg	Rise mg	Tocopherol mg per diem	Administered days*	Clinical status
101	M	48	.32	—	—			
102	M	51	.63; .50	—	—			
5	M	47	.69	—	—			
8	M	39	1.15	1.36	0.21	75	1	
103a	M	50	.56	0.20	—0.36	75	8	
104a	F	56†	.40	0.66	0.26	75	4	
			.70	0.74	0.04			
105a	M	42	1.16; .90	0.82	—0.08	100	4	
106a	M	52	.66	0.78	0.12	100	7	
13	M	38	.67	0.90	0.23	100	5	
107	F	37	.74; .80	1.25	0.45	100	4	++
9	F	50	.61	1.20	0.59	100	4	
			.73	0.99	0.27			
106b	M	52	[.78]‡	1.10	0.32	200	9	++
104b	F	56	[.66]‡	1.02	0.36	200	5	
108	F	31	.52; .49	0.90	0.41	200	6	+
105b	M	42	[.82]‡	1.54	0.72	200	6	
109	M	38	.59	1.49	0.90	200	5	+
103b	M	50	[.20]	1.29	1.09	200	6	
110	M	55	.97; .90	2.08	1.18	200	12	
			.66	1.37	0.73			
10	M	43	.55; .53	0.93	0.40	340	8	
6	F	39	.80	2.26	1.46	340	5	++
			.67	1.60	0.94			
11	M	50	.69; .88	[1.70]	[0.82]	[740]	[3]	
				2.00	1.12	740	10	++
2	M	41	—	1.68	—	740	67	++
1	F	48	—	1.71	—	740	23	
7	F	58	—	1.79	—	740	22	
3	M	32	—	2.07	—	740	69	
8	M	39	—	2.14	—	740	28	
			.88	1.90	1.12			
Average of all cases:			0.67	—	—			

The case numbers below 100 correspond to the numbers in Table II of our previous publication.[1]

* Number of days indicates time elapsed from the beginning of administration to the day of determination "after treatment," but treatment was actually often continued beyond that date.

† Diagnosis of No. 104 not definite.

‡ The figures in brackets have been excluded from computations of the total average. In case of duplicate determinations, separated by a few days, the first one has been disregarded in averaging.

\+ = No evidence of progression while under observation.

++ = Temporary improvement or recession of signs and symptoms.

grouped according to the strength of the daily doses. Fig. 1 shows that 75 mg per diem leave the average serum level unaffected. 100 mg a day cause a rise of 0.27 mg in cases of amyotrophic lateral sclerosis, but amounts of 200 mg double the tocopherol titer to

TABLE II.
Serum Tocopherol in Miscellaneous Cases Before and After Tocopherol Administration.

No.	Sex	Age	Diagnosis	Tocopherol content of 100 ml serum: Before treatment mg	After treatment mg	Rise mg	Tocopherol mg per diem	Administered days*
201	F	31	Myopathy	.60; .64	—	—		
202	M	50	Lumbo-sacral radiculitis	.63	—	—		
203	M	42	Lateral sclerosis	.90	—	—		
204	M	55	Lumbo-sacral radiculitis	1.07	—	—		
				.81				
205	M	8	Myopathy	.35; .33	0.50	0.17	50	11
208a	F	32	"	.88	0.69	—0.19	75	4
206a	M	21	Normal	.54	0.59	0.05	75	5
207	M	18	Myopathy	.25	0.42	0.17	75	5
				.56	0.57	0.01		
209a	M	24	"	.80; .51	0.43	—0.08	100	8
210	M	22	"	.46; .79	1.00	0.21	100	5
				.65	0.72	0.07		
211	M	18	"	.52	0.35	—0.17	150	11
206b	M	21	Normal	[.59]‡	0.50	—0.09	150	5
212	F	58	Myopathy	.54	1.60	1.06	150	8
				.53	0.82	0.27		
209b	M	24	"	[.43]‡	0.40	—0.03	200	8
208b	F	32	"	[.69]‡	0.93	0.24	200	4
213	M	33	"	.48	1.49	1.01	200	17
				.48	0.94	0.41		
214	F	47	Diffuse calcinosis	.40; .49	1.32	0.83	300	7
Average of all cases:				.61	—	—		

values approaching those observed previously[1] with daily doses of 340 mg and 740 mg. The respective increases with 100-300 mg per diem in the miscellaneous group were erratic and their averages distinctly lower than in amyotrophic lateral sclerosis. In 4 individuals (Nos. 103-106) the rise was determined after a first period of 75-100 mg per diem and a second period of 200 mg per diem. The response rose from —0.36 to +1.09, from 0.26 to 0.36, from —0.08 to +0.72 and from +0.12 to +0.32 respectively. Similar experiments on one normal subject (206) and 2 cases with muscular dystrophy (208 and 209) yielded equivocal results.

A comparison of daily dosage and rise in blood level with the temporary improvement, observed during tocopherol administration in amyotrophic lateral sclerosis, shows that such beneficent effects occurred in one of 8 cases receiving 100 mg p.d. or less and in 6 out of 9 cases receiving 200 mg or more. None of 6 cases whose blood level had decreased, or increased by less than 0.40 mg/100 ml serum showed any favorable effect, whereas 7 of 11 cases showed such temporary effects concomitantly with a rise of serum tocopherol by more than 0.40 mg/100 ml. These findings suggest that any further therapeutic trials with oral tocopherol administration in amyotrophic lateral sclerosis should be based on repeated daily dosage of 200 mg or more.

The miscellaneous group is not sufficiently

large to correlate the nature of the response of the blood level with individual disease entities.

We could not detect tocopherol in any of 6 specimens of spinal fluid from amyotrophic lateral sclerosis and other patients.

Summary. In continuation of previous studies, the tocopherol level in the serum of patients with amyotrophic lateral sclerosis and with miscellaneous myopathies was found to average 0.67 and 0.61 mg/100 ml respectively. The response to daily oral administration of 75 to 740 mg tocopherol runs parallel to the dosage and attains levels of more than 2.0 mg/100 ml serum. Temporary favorable effects on the clinical status are only found with doses over 200 mg tocopherol per diem. The spinal fluid does not contain tocopherol.

14236

Heparin and the Agglutination of Platelets *in Vitro.*

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Heparin inhibits both the coagulation of the blood and the agglutination of platelets. A much higher concentration, however, is required for the latter. Solandt and Best[1] have shown that over 300 mg per kg of body weight must be injected intravenously to prevent the intravascular clumping of platelets. The object of the present study was to determine the minimum amount of heparin that is required for the prevention of platelet agglutination *in vitro.*

Blood was drawn into a dry sterile syringe, and immediately distributed in 1 cc amounts to a series of small test tubes containing 0.1 cc of heparin solution (Roche Organon Liquaemin*) of varying concentrations as recorded in the table. After thorough mixing, the blood was drawn to the 0.5 mark in a red blood cell pipette and the pipette filled with 0.85% sodium chloride solution. The platelet count was made in the usual manner.

A concentration of more than 0.1 mg (or 10 Toronto units) per cc of human blood is required to prevent clumping of platelets. No decrease in the platelet count by heparin as reported by Copley and Robb[2] was observed. Why so much more heparin is required for the prevention of platelet agglutination than for inhibiting coagulation is not clear. Nevertheless, it is well recognized that a definite relationship exists between these two processes. An additional observation in support

TABLE I.
Influence of Heparin on the Platelet Count *in vitro.*

Amt of heparin per cc of blood	Platelet count of human subjects			State of platelets
	I	II	III	
None*	337,000	312,000	278,000	No clumping
0.1 mg	360,000	320,000	286,000	Definite clumping
0.25	372,000	300,000	270,000	No clumping
0.5	320,000	208,000	264,000	" "
1.0	348,000			" "

* 0.1 cc of 3.8% sodium citrate was used.

1 Solandt, D. Y., and Best, C. H., *Lancet*, 1940, **1**, 1042.

* The Liquaemin was kindly supplied by Edward A. Wickham of Roche Organon, Inc., Nutley, New Jersey.

2 Copley, A. L., and Robb, T. P., *Am. J. Clin. Path.*, 1942, **12**, 416.

of this may be cited. The platelets of blood obtained from animals whose prothrombin has been drastically reduced by feeding dicumarol show no tendency to agglutinate in the absence of an anticoagulant. A typical experiment was as follows: Dicumarol was given to a rabbit daily until the prothrombin time was 7 minutes. Platelet counts were made using for one sample 3.8% sodium citrate as the diluent, and for the other 0.85% sodium chloride solution. The platelet count using saline solution was 250,000, and with sodium citrate solution was 280,000. No clumping in either was observed.

14237 P

Rate of Emptying of Biliary Tract Following Section of Vagi or of All Extrinsic Nerves.*

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Previous experiments have demonstrated that section of the specific nerves to the choledochoduodenal junction in the cat so retards emptying of the gall bladder that double the time is required to empty half its contents after a meal of egg-yolk. Subdiaphragmatic section of the left vagus has approximately the same effect but when the right vagus is cut the time is tripled. Section of all splanchnics produces little change.[1] The evidence suggests that severance of the right vagus removes not only the fibers which activate the gall bladder but those which release the sphincter of Oddi by inhibiting the local nerve net that maintains its tonus between meals.

Surprisingly, a continuation of these experiments has shown that severance of *both* vagi, or complete denervation of the biliary tract (vagi and splanchnics), has no retarding effect on the rate of emptying, *i.e.*, gall bladder and sphincter respond to hormone-producing food at least as fast as in the controls.

Since it is known that section of one vagus does not affect the rate of emptying of the stomach[2] but that section of both vagi relaxes the pyloric sphincter and decreases the initial time required for passage of food into the duodenum,[3] the rate of emptying of the denervated biliary tract may be explained on the basis that loss of vagus effect on the biliary musculature is compensated for by increased hormonal action resulting from faster discharge of hormone-producing food into the duodenum. Contrary to some earlier reports on section of nerves to the intestine[4] it can be stated that severance of all extrinsic nerves to the sphincter of Oddi, although resulting in destruction of preganglionic fibers, does not

TABLE I.
Rate of Emptying of Gall Bladder After Egg-yolk.

Nervous pathway interrupted	No. of animals	Initial tonus (min)	Oil enters cystic duct (min)	½ contents gall bladder discharged (min)	⅔ contents discharged (min)
Lipiodol controls (DuBois and Hunt, '32)	12	9	20	70	104
Both vagi	12	10	22	64	89
Vagi and splanchnics	11	9	26	68	87

* Aided by grants from the Graduate Research Funds of the University of Minnesota.

1 Johnson, Frank E., and Boyden, Edward A., *Surg., Gyn., and Obst.*, 1943, **76**, 395.

2 McSwiney, B. A., *Physiol. Rev.*, 1931, **11**, 478.

3 Meek, Walter J., and Herrin, Raymond C., *Am. J. Physiol.*, 1934, **109**, 231.

4 Evans, C. L., *Physiol. Rev.*, 1926, **6**, 358.

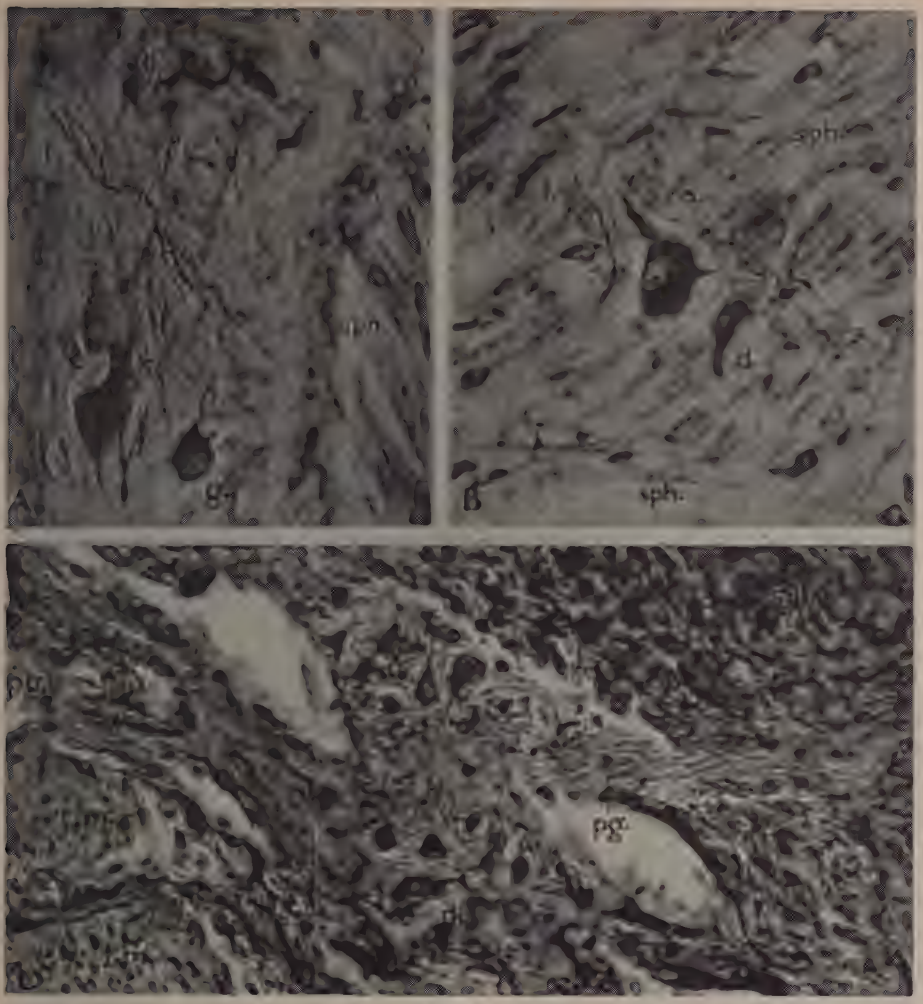

FIG. 1.

A, B. Photomicrographs of sphincter of Oddi (sph.) 11 days after peripheral connections of left celiac and central connections of right celiac ganglion have been severed (Cat No. 52; Bodian technic; × 352). Note undegenerated extrinsic nerve fibers (*f*) in vicinity of ganglion cells (*g*). *a, d,* axon and dendrites.

C. Photomicrograph of tangential section of major papilla 12 days after both vagi and peripheral connections of both celiac ganglia have been severed (Cat No. 72; Bodian technic; ×300). Note absence of extrinsic nerves but persistence of peripheral ganglion cells (*g*) and their postganglionic fibers (*p.g.*). The latter pass both to the tunica muscularis (*t.m.*) and to the sphincter muscle (*sph.*).

cause degeneration of the ganglion cells and postganglionic fibers (Fig. 1 C). This local nerve net may be presumed to be the apparatus which continues to respond to hormone-producing substances.[5] The accompanying figures, showing sections stained by the Bodian technic (C. Van Buskirk), are believed to be the first published photomicrographs of the intrinsic nerve net of the sphincter of Oddi.

[5] Boyden, Edward A., Bergh, George S., and Layne, John A., *Surgery*, 1943, **13**, 723.

14238

Fat Oxidation in Experimental Animal Diets.*

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The large number of deleterious effects caused by the ingestion of oxidized fat, which have been reported from numerous laboratories, will be reviewed in a forthcoming publication.[1] In many cases improper stabilization of experimental diets may have resulted in the inadvertent feeding of rancid fats to test animals. Although it is impossible to tell how frequently experimental results have been unknowingly influenced by this factor more care should be exercised by investigators in preventing fat deterioration. The seriousness of fat rancidification was again brought to the attention of this laboratory some time ago when it was found that rats receiving a normal balanced diet containing the pure B vitamins in place of the customary yeast, cod liver oil, and lard, failed to grow and ultimately died. Replacement of the lard with other fats such as hydrogenated vegetable oils, corn oil, and butterfat provided an adequate diet for growth. If the pure B vitamins were replaced by yeast, normal growth resulted. On further investigation it was found that in the absence of yeast, the lard and cod liver oil rapidly became oxidized and exerted a deleterious effect.

As it is a common practice among nutrition investigators to prepare experimental diets and keep them at room temperatures over periods of several weeks it was considered advisable to have some knowledge of the rates of deterioration of fats incorporated in different food mixtures.

Experimental. Dietary mixtures were compounded in 200 g lots as outlined in Table I. These mixtures were placed in glass bottles and stored in the dark at room temperature. Once each week 25 g samples were removed and extracted with ligroin (B. P. 30-60°C.). After removal of the solvent *in vacuo,* peroxide values were determined.[2] These peroxide values in milliequivalents per 1000 g of fat are plotted in Fig. 1, 2, and 3. The peroxide values of the individual fats at the time of mixing were: wheat germ oil, 7.3; cod liver oil, 18.6; steam rendered lard, 2.4; bland (stabilized) lard, 0.3; vegetable (Wesson) oil, 14.2; and butterfat, 0.0.

Results. It is shown in Fig. 1 that the lard in diet mixtures not containing yeast reached the end of the induction period within 2-3 weeks. The stability of this fat was not greatly influenced by the presence of the pure B vitamins, but addition of yeast prolonged the induction period beyond 8 weeks.† When mixed with Cellu Flour (Fig. 3, Diet 19) the induction period of lard was much longer than when it was mixed with other dietary constituents except yeast (Fig. 1, Diet 5). It seems that a prooxidant, probably the salts, must promote oxidation in the food mixture.

In Fig. 2 are given the oxidation curves for several fats mixed in diets containing the pure B vitamins and cod liver oil, with or without wheat germ oil. The induction period of a lard-containing diet without either cod liver oil or wheat germ supplements (Diet 2) was prolonged slightly by the addition of cod liver oil (Diet 7) and to a greater extent by cod liver oil together with wheat germ oil

* This work was aided by a grant from the Rockefeller Foundation.

1 Burr, G. O., and Barnes, R. H., *Physiol. Rev.*, in press.

2 King, A. E., Roschen, H. L., and Irwin, W. H., *Oil and Soap,* 1933, **10**, 105.

† Since the completion of this study György and Tomarelli (György, P., and Tomarelli, R., *J. Biol. Chem.*, 1943, **147**, 515) have presented evidence of the antioxidant properties of yeast and the lack of antioxidant activity of the known B vitamins with the exception of *p*-aminobenzoic acid, in mixtures of corn starch, butter yellow (N,N-dimethylaminoazobenzene), and crude linoleic acid (iodine value 126-140).

TABLE I.
Dietary Mixtures.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Salts	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
Yeast	+							+											
Pure B Vitamins		+				+	+		+	+	+	+	+	+	+	+			
B Vitamins minus Choline			+																
Choline				+															
Cellu Flour																	+	+	+
Mineral Oil															+	+			
Wheat Germ Oil						+		+	+		+		+		+		+		
Cod Liver Oil						+	+	+	+	+	+	+	+	+	+	+	+	+	
Type of Fat	S. lard	S. lard	S. lard	S. lard	S. lard	S. lard	S. lard	S. lard	B. lard	B. lard	Veg. oil	Veg. oil	B. fat	B. fat	—	—	S. lard	S. lard	S. lard

When present in a given mixture the ingredients were added per 100 g of diet in the amounts specified below: Casein (Land O'Lakes, 100 mesh) 20 parts; sucrose (pure cane sugar) sufficient to make a total of 100 g; salts (modified Hubbell, Mendel and Wakeman[3] $CaCO_3$, 543; $MgCO_3$, 25; $MgSO_4 \cdot 7H_2O$, 16; NaCl, 690; KCl, 112; KH_2PO_4, 212; $FePO_4$, 20.5; KI, 0.08) 4 parts; yeast (Anheuser Busch strain G) 6 parts; pure B vitamins (thiamin, 1 mg; riboflavin, 375 μg; nicotinic acid, 2 mg; pyridoxine, 500 μg; pantothenic acid, 2.8 mg; choline, 200 mg; 2-methyl 1-4 naphthoquinone, 40 μg); B vitamins minus choline (the above vitamins without choline); choline, 200 mg; Cellu Flour (Chicago Dietetic Supply House) sufficient to make a total of 100 g; mineral oil (heavy U.S.P.) 18 parts; wheat germ oil (Viobin) 1 part; cod liver oil (U.S.P.) 2 parts; steam lard (Hormel) 18 parts; bland lard (Swift's, contains some hydrogenated fat and 0.1% gum guaiac) 18 parts; vegetable oil (Wesson oil) 18 parts; butterfat (commercial butter melted and centrifuged to separate fat) 18 parts.

(Diet 6.) Bland lard which contains the antioxidant gum guaiac, had a definitely prolonged keeping time, but was not appreciably affected by the inclusion of wheat germ oil (Diets 10 and 9). The induction period of the butterfat diet was prolonged to some extent by wheat germ oil (Diets 14 and 13) but

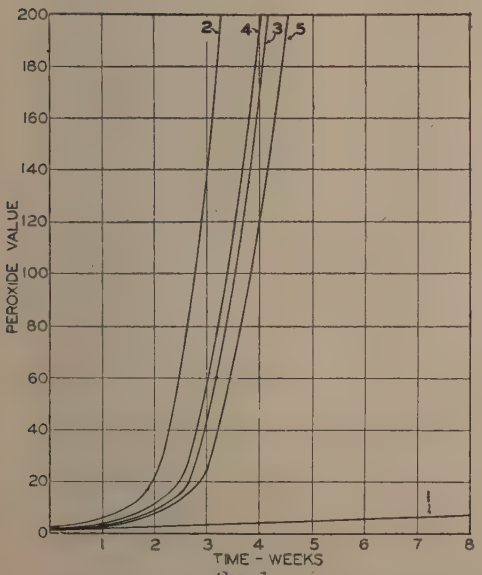

FIG. 1.

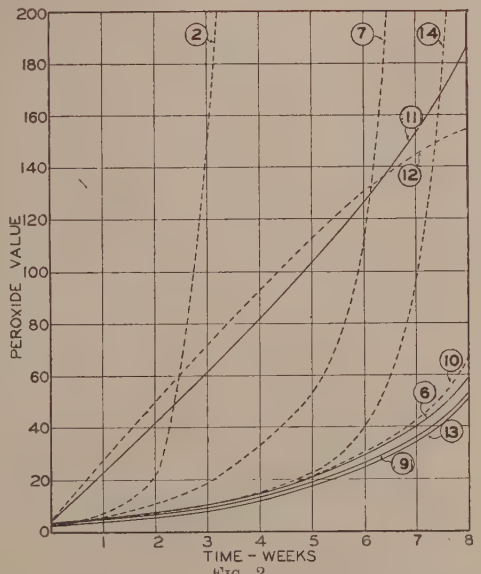

FIG. 2.

the cottonseed oil diet which had a steady peroxidation from the start was not protected (Diets 12 and 11).

In the absence of other fats the mixture of cod liver oil and wheat germ oil oxidized much

[3] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

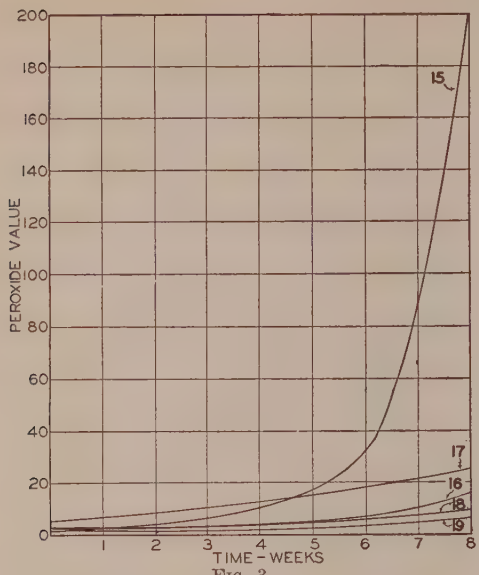

FIG. 3.

more rapidly than cod liver oil alone (Fig. 3, Diets 16 and 15).

Data which are not included in the graphs showed that after a 6-weeks storage of yeast-containing diets at room temperature (Diet 8 with other fats substituted) none of the following fats had developed peroxide values above 15: lard Swift's Silverleaf, lard Cudahy-Rex, lard Swift's-Bland with gum guaiac, corn oil (Mazola), hydrogenated vegetable oil (Crisco), or butterfat.

Discussion. The rancidification of lard in a diet which did not contain yeast was very rapid. With the addition of cod liver oil and wheat germ oil the rate of accumulation of peroxides was considerably less. However it is well known that both of these oils are themselves unstable and that cod liver oil rapidly destroys vitamin E in mixed diets. It is certainly not good practice to incorporate these 2 fat soluble vitamin sources in the diet mixture, especially when yeast is not present. For purposes of controlling nutrition factors it is frequently advocated that yeast supplements be fed separately from the rest of the dietary mixture. When this method of yeast administration is not necessary a prolonged keeping time of the food fat and a probable protection of readily oxidizable vitamins is afforded by the direct incorporation of the yeast in the diet. It is also advantageous to employ other methods of stabilization. Lard containing 0.1% gum guaiac had a somewhat longer induction period. Storing of diets at low temperatures is to be recommended. Diet No. 2 which contains lard and the pure B vitamins had a peroxide value of 12 after approximately 3 weeks in an ice box at a temperature of 12°C. When stored at room temperature for the same time the lard of this diet had a peroxide value greater than 100.

The variation in the keeping quality of different fats and the influence of other diet components on the stability of fat makes careful control of rancidification imperative in nutrition studies. The inadvertent destruction of dietary essentials and the possible independent toxicity of rancid fat are factors that might confuse the interpretation of many diet experiments.

Summary. Studies on the keeping quality of fats in different food mixtures have indicated certain prooxidant and antioxidant characteristics among the dietary components. The relative keeping time of fats added to various dietary mixtures is given and the importance of careful preservation of experimental diets is stressed.

14239

Effect of Acetylation on Specificity of Pepsin.

VINCENT HOLLANDER. (Introduced by F. C. Koch.)

From the Department of Biochemistry, University of Chicago.

The action of crystalline pepsin on synthetic substrates such as carbobenzoxy l-glutamyl l-tyrosine (C.G.T.) exhibits at least two well defined differences from its action on a protein like hemoglobin.[1] First, the action of pepsin on hemoglobin has a maximum rate at pH 2, while the action on C.G.T. has a maximum rate at pH 4. Secondly, it requires high concentrations of pepsin to effect rapid hydrolysis of C.G.T.

Since the action of pepsin on a protein can be quantitatively altered by acetylation of the enzyme, it was thought desirable to test the effect of acetylation on the action of C.G.T. and to compare this with the action on the typical protein substrate, hemoglobin.

This was done with 3 types of pepsin preparations. First, the crystalline N-acetyl derivative was prepared by ketene acetylation of crystalline pepsin. The crystalline pepsin was prepared from commercial Cudahy 1:10,000 pepsin by the method of Northrop.[2] The preparation and crystallization of the N-acetyl derivative was done according to the description of Herriott and Northrop.[3] This preparation is as active on hemoglobin as is unacetylated pepsin. Second, a series of more highly acetylated pepsins ranging in activity from 60% to 100% of that of regular crystalline pepsin were prepared by further treatment with ketene. No attempt was made to crystallize these preparations. Third, preparations were made by acid hydrolysis of the 0-acetyl groups in a 50% active preparation which causes the enzyme to regain its activity[3,4] as a result of an increase in the number of free phenolic hydroxyl groups of tyrosine.

The action of the enzyme on hemoglobin was determined according to the method of Anson and the results recorded in terms of hemoglobin units of specific activity.[5] The action on C.G.T. is recorded in terms of the percent hydrolysis after 24 hr incubation at 40°C of a solution .035 M in C.G.T. and .05 M in acetate buffer adjusted to pH 4.0 with dilute sodium hydroxide using a glass electrode. The extent of hydrolysis was determined by the volumetric method of Van Slyke for amino nitrogen and corrected for amino nitrogen in the enzyme preparations themselves. The results on the first two types of preparations are recorded in Table I.

To test the effect of acid hydrolysis of 0-acetyl groups, pepsin which had been acetylated to about 50% of its former activity was treated with normal sulfuric acid at 2°C. The results are recorded in Table II.

These results show that mild acetylation of pepsin has no influence on the activity of the enzyme towards C.G.T.; that more drastic acetylation inhibits this activity towards C.G.T. in the same manner as it does the activity towards hemoglobin. These experiments support the conclusion[3] that free amino groups in the enzyme are unnecessary for the action of pepsin and that free phenolic hydroxyl groups are necessary. If the percent hydrolysis of C.G.T. is plotted against the concentration of pepsin (in the range used in these experiments) a linear relationship is obtained. If points for the acetyl pepsin experiments are located on this graph by using, instead of pepsin concentration, the function acetyl pepsin concentration $\times \frac{\text{Sp. activity after acetylation}}{\text{Sp. activity before acetylation}}$ the points fall on the line. This is consistent with the interpretation that the hydrolysis of C.G.T. and hemoglobin by pepsin are similar processes. This is also consistent with the find-

[1] Fruton, J. S., and Bergmann, Max, *J. Biol. Chem.*, 1939, **127**, 627.

[2] Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.

[3] Herriott, R. M., and Northrop, J. H., *J. Gen. Physiol.*, 1934, **18**, 13.

[4] Herriott, R. M., *J. Gen. Physiol.*, 1935, **19**, 283.

[5] Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.

TABLE I.
Comparative Activities of Pepsin and Acetylated Pepsins.

Preparation	% hydrolysis C.G.T.	Specific activity on hemoglobin
Crystalline N-acetyl pepsin	55 a	.22
" " "	55 a	.22
2X cryst. pepsin	54 a	.23
" " "	55 a	.23
" " " acetylated for 2 hrs	52.5b	.23
3 "	44.6b	.22
4 "	43.2b	.21
7 "	41.3b	.15

a—1.2 mg pepsin N/ml. b—1.0 mg pepsin N/ml.

TABLE II.
Comparative Activities of 50% Active Pepsin Before and After Hydrolysis.

Hrs acid hydrolysis	Conc. pepsin used on synthetic substrate in mg protein/ml	% hydrolysis of C.G.T.	Specific activity on hemoglobin
0	0.8	36	.12
0	1.2	40.5	.12
48	0.8	39	.16
48	1.2	45	.16
100	0.8	45	.21
100	1.2	51.5	.21

ings of Tracy and Ross[6] that malonylation of pepsin with carbon suboxide does not effect the specificity of the enzyme.

The difference between C.G.T. and hemoglobin with respect to pH optima of hydrolysis is perhaps ascribable to the mechanism of the enzyme reaction. Bergmann[1] demonstrated the importance of carboxyl groups in synthetic substrates for pepsin; and Tracy and Ross,[6] found that the introduction of carboxyl groups into serum albumin by malonylation increases the rate and extent of peptic activity. If one assumes that pepsin catalyses the hydrolysis of appropriate substrate molecules with intact carboxyl groups (as against carboxyl ions) the difference in pH optima can be explained by the existence in the protein substrate of stronger acidic groups (which require a lower pH for their conversion to carboxyl groups) than in the peptide. The assumption is consistent with, but not uniquely demonstrated by, the data of Northrop,[7] who showed that the rate of peptic digestion runs parallel to the titration curve of protein substrates. Further data is of course necessary to test this point.

The author wishes to thank Professor F. C. Koch of the Department of Biochemistry of the University of Chicago for his advice and encouragement.

Summary. Acetylation of the free amino groups of pepsin does not affect the hydrolysis of the peptide carbobenzoxy l-glutamyl l-tyrosine. Further acetylation decreases the activity towards this substrate. Acid hydrolysis of 0-acetyl, leaving N-acetyl groups intact, restores the activity of the enzyme to its former value.

[6] Tracy, A. H., and Ross, W. F., *J. Biol. Chem.*, 1942, **146**, 63.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1922, **5**, 263.

14240

Anti-Rachitic Activity of Vitamin-D_3-Precursors in the Rat.

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In a preceding paper[1] we have found that subcutaneous injection of 7-dehydro-cholesterol shows an anti-rachitic activity only if the previously depleted rats also receive ample irradiation. Further studies of the anti-rachitic activity of other cholesterol derivatives are submitted in this report.

In the synthesis of 7-dehydro-cholesterol from cholesterol, 7-keto-cholesterol and 7-hydroxy-cholesterol* are important intermediaries. It was, therefore, considered of interest to investigate whether or not any evidence could be found for the ability of the animal body to form 7-dehydro-cholesterol from its *in vitro* precursors, namely, 7-keto-cholesterol and 7-hydroxy-cholesterol. Such a possibility has been indicated by Wintersteiner and Ritzman[2] and Rosenberg.[3] Furthermore, Hazlewood[4] and McPhillamy[5] isolated 7-hydroxy-cholesterol from liver and from serum. Wintersteiner and Ritzman,[2] and Wintersteiner and Bergstrom[6] thought that the 7-hydroxy-cholesterol isolated by the above workers might possibly be an artefact produced by accidental oxidation during its isolation. This possibility seems to be eliminated by the more recent work of Hazlewood.[7]

It was thought that the injection of solutions of 7-keto-cholesterol and 7-hydroxy-cholesterol into rachitic rats on a depletion diet followed by exposure of the rats to ultraviolet irradiation might indicate whether the animal body is capable of converting these two precursors into 7-dehydro-cholesterol.

The following experiments were therefore undertaken:

7-dehydro-cholesterol was prepared from cholesterol by the method of Windaus[8] and the product was repeatedly recrystallized from methyl alcohol in darkness. The α-epimer of 7-hydroxy-cholesterol was prepared by saponification from the dibenzoate according to Windaus[8] and was recrystallized several times from petrol ether. The 7-keto-cholesterol was prepared by saponification from the keto-cholesterol-acetate and recrystallized from methyl alcohol (m.p. 166 to 167° C Σ_1 238 = 12760). These 3 sterol compounds were dissolved in propylene-glycol U. S. P., using 5.3 mg of the purified substance for 50 cc of propylene-glycol. The solutions were kept in a dark refrigerator.

For each group of experiments 7 fully depleted rats (see U.S.P. XII, p. 640) were used.

Group 1 was injected subcutaneously every other day with 0.2 cc of 7-dehydro-cholesterol solution.

Group 2 was injected subcutaneously every other day with 0.2 cc of 7-hydroxy-cholesterol.

Group 3 received subcutaneously every other day 0.2 cc of 7-keto-cholesterol solution.

All 3 groups were irradiated daily for 4 minutes with a 2 arc therapeutic lamp at a

1 Geiger, E., and Lassen, S., Proc. Soc. Exp. Biol. and Med., 1942, **52**, 11.

* This compound is usually not regarded as a pro-vitamin in a strict sense as irradiation of this substance (Bills and MacDonald[10]) shows only a very slight anti-rachitic activity and there is, furthermore, no indication that the material used for irradiation in those experiments did not contain 7-dehydro-cholesterol as impurity.

2 Wintersteiner and Ritzman, *J. Biol. Chem.*, 1943, **136**, 697.

3 Rosenberg, H. R., *Chemistry and Physiology of the Vitamins*, Interscience Publ., 1942.

4 Hazlewood, G. A. D., *Biochem. J.*, 1939, **33**, 709.

5 McPhillamy, H. B., *J. Am. Chem. Soc.*, 1940, **62**, 3518.

6 Wintersteiner and Bergstrom, *J. Biol. Chem.*, 1941, **141**, 597.

7 Hazlewood, G. A. D., *Biochem. J.*, 1942, **36**, 389.

8 Windaus, A., Lettre, H., and Schenck, F., *Ann.*, 1935, **98**, 520.

TABLE I.

Test No.	Group of 7 rats	Injected	Avg Healing
Test A	I	7-dehydro-cholesterol	1.2
	II	7-hydroxy- "	2.0
	III	7-keto- "	1.1
Test B	I	7-dehydro-cholesterol	0.86
	II	7-hydroxy- "	1.8
	III	7-keto- "	1.75

distance of 12 inches. The animals were also daily exposed to daylight for 3 hours. Otherwise the rats were kept during the whole experiment on a rachitogenic diet in a dark room. The injection of the sterols was always performed after the irradiation. After 8 days, the rats were killed and the degree of calcification in the tibiæ was evaluated by the line-test. (Table I).

The results of Table I, Test A, show that the injection of 7-hydroxy-cholesterol gave greater calcification than the injection of 7-dehydro-cholesterol did and that the 7-keto-cholesterol had about the same activity as 7-dehydro-cholesterol. In order to reduce any experimental error, we repeated this experiment using newly prepared sterol solutions. The results of this series are listed in Table I under Test B. The repetition of the experiments confirmed the results of Test A, showing the 7-hydroxy-cholesterol and 7-keto-cholesterol injected subcutaneously into rats subsequently irradiated was more effective than the injection of 7-dehydro-cholesterol, but this time the 7-keto-cholesterol was as effective as 7-hydroxy-cholesterol.

If we assume that the 7-keto-cholesterol and 7-hydroxy-cholesterol were transformed in the body of the experimental animals to 7-dehydro-cholesterol in the same general way as *in vitro* and that this substance is finally activated by our irradiation procedure, it is difficult to explain why the precursor is more active than the 7-dehydro-cholesterol itself. We are, therefore, forced to conclude that either the keto and hydroxy compounds are more easily absorbed from the place of injection (subcutis) than the 7-dehydro-cholesterol and are conveyed faster to the specific tissues (liver, skin, or bones) where they exert their activity or that the keto and the hydroxy compounds in the body are transformed to Vitamin D or pro-vitamin D in a way which is entirely different from the *in vitro* procedure. Further experiments are in progress to determine which one of the two assumptions is correct.

In the *second* group of experiments we compared the anti-rachitic activity of the above mentioned 3 sterols when given *per os* and by injection. The mode of procedure is that used above. Seven or 14 D-depleted rats were used in each group and the animals were irradiated daily as in the experiments mentioned before. 0.1 cc of the propylene glycol solution of the same concentration as used for the subcutaneous injection was administered daily *per os* following the irradiation of the animals, so that the total amount of the sterols administered to each rat during the experiment was the same as in the earlier experiments using the injection method.

TABLE II.

Group No.	Rats in Group	Treatment	Avg Healing
I	14	Irradiation and *per os* daily 0.1 cc 7-dehydro-cholesterol	0.39
II	7	Irradiation and *per os* daily 0.1 cc 7-hydroxy-cholesterol	0.64
III	7	Irradiation and *per os* daily 0.1 cc 7-keto-cholesterol	0.29

The experiments of Table II show that *per os* administration of the 3 sterols investigated was less effective than the subcutaneous injection of the same sterols. This may be attributed to a diminished resorption of these compounds from the intestinal tract. In the foregoing paper[1] we have proved that there is no essential difference in the curative effect of *activated* 7-dehydro-cholesterol whether it is given the rats subcutaneously or *per os*. This seems to indicate that the intestinal resorption of the so-called D_3-pro-vitamins is very limited in comparison with that of the irradiated product.

This result is in conformity with the observations of Schoenheimer *et al.*[9] who showed that ergosterol is likewise less readily

[9] Schoenheimer, Behring and Gottberg, *Z. Physiol. Chem.*, 1932, **208**, 77.

[10] Bills, C. E., *Cold Spring Harbor Sym. Quant. Biol.*, 1935, **3**, 328.

absorbed from the intestinal tract than Vitamin D_2, produced from this pro-vitamin by irradiation.

Summary. 1. 7-hydroxy-cholesterol when given subcutaneously to rats, that are subsequently exposed to ultra-violet irradiation has a higher anti-rachitic effect than 7-dehydro-cholesterol injected into rats under identical conditions. 2. 7-keto-cholesterol when given subcutaneously to rats that are subsequently exposed to ultra-violet irradiation has equal or higher-anti-rachitic effect than 7-dehydro-cholesterol. 3. The *per os* introduction of 7-keto-cholesterol, 7-hydroxy-cholesterol and 7-dehydro-cholesterol into rats subsequently irradiated produces a lesser anti-rachitic effect than the subcutaneous injection of similar amounts of the sterols under otherwise identical conditions. Activated 7-dehydro-cholesterol forming an exception as shown in a previous report.

14241

A Sensitive Test for Fox Encephalitis Virus.

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Work with the virus of fox encephalitis has been hampered by the failure of this virus to cause visible illness or death in all infected animals. In a small series of from 5 to 10 foxes or dogs, evidence of infection may be observable in from 0 to 100% of animals after the intramuscular or intracerebral injection of adequate doses of living virus. Such inconstant results make accurate titrations of virus or antiserum, or the test of the efficacy of a vaccine, so costly as to be impracticable.

The following experiments have been conducted in developing a sensitive test for fox encephalitis virus. It was believed that foxes that failed to show symptoms of infection after the injection of virus underwent inapparent infections.[1] Inasmuch as cells infected with the fox encephalitis virus develop characteristic, large, intranuclear inclusions, an attempt was made to place the virus in contact with susceptible cells that could be subsequently examined histologically. It is known that this virus attacks vascular endothelium, reticulo-endothelium of the liver, spleen, and lymph nodes, histiocytes, and certain specialized cells such as ependymal and hepatic cord cells.

In our laboratory, fox encephalitis virus is routinely preserved as a 20% suspension of homogenized fox brains in 50% neutral glycerin. One such suspension, Lot 94, mixed with an equal volume of a similar suspension of liver from infected foxes was used in the first experiment. Just before injection, the tissue suspensions were diluted with 9 volumes of isotonic NaCl solution.

A 3-fold approach to the problem was made by searching for inclusion bodies in (1) tissues at the site of subcutaneous and intramuscular injections, (2) lymph nodes draining such regions, and (3) cells of the eye after intra-ocular injection. Virus was injected into each of the 4 legs and into the floor of the mouth of 5 foxes. Tissue from the sites of subcutaneous and intramuscular injections (1 or 2 cc of 2% virus suspension) was removed at intervals of from 1 to 5 days and examined for inclusion bodies. The results were consistently negative. It is probable that inclusions develop in such areas but are too hard to find to be of value in detecting small amounts of virus.

From the same animals, lymph nodes draining the regions in which the inoculations had been made were removed at intervals of from 1 to 5 days. Grossly, a few of the lymph nodes were enlarged, edematous and hyperemic; others were normal. As shown

[1] Green, R. G., Katter, M. S., Shillinger, J. E., and Hanson, K. B., *Am. J. Hyg.*, 1933, **18**, 462.

TABLE I. Development of Fox Encephalitis Inclusion Bodies in Lymph Nodes and Eyes of Foxes.

Days after injection	Fox No.	Lymph nodes: Popliteal L	Popliteal R	Inguinal L	Inguinal R	Iliac L	Iliac R	Axillary L	Axillary R	Supraclavicular Submaxillary L	Supraclavicular Submaxillary R	Eyes L	Eyes R
1	30678	Biopsy	0	0				0					++
	30682	"			0								
2	30678	Killed	0	0		++++	++	0		0		++	
	30679	Biopsy											++
	30682	Killed	0	0	0*	0		0			0	++	
3	30679	Biopsy	0	0				++		0	++	+++	
	30680	"	++	0				+++		0	+		+++
	30681	"											++
4	30679	Killed	+	+++		++++	+++	++		0	++		
	30680	Biopsy										++++	
	30681	"	0	0				+++		0	+	+++	
5	30680	Died	++		0*	++++	++++		+++				
	30681	Killed	+++	+	++	++++	+++		++	++	+	0	

* Inflammation but no inclusion bodies. Ciphers indicate that no inclusion bodies were seen. Plus signs indicate relative abundance of inclusion bodies.

in Table I, inclusions were found regularly in lymph nodes from the axillary and iliac regions. It is probable that the lesser degree of success in finding inclusion bodies in lymph nodes from other areas could be ascribed principally to our failure to remove the correct node. The results indicated that examination of regional lymph nodes after injection of virus would prove a successful method of detecting fox encephalitis virus. This method was not studied further only because intra-ocular injection was found to be even more satisfactory. However, it seems probable that in the case of some other viruses in which silent infections are an obstacle to investigation, utilization of lymph nodes in this manner will be of value.

All 5 foxes used in the preceding studies were also inoculated in the eye. Under ether anesthesia a hypodermic needle was inserted through the sclera near the corneal margin and was passed forward into the anterior chamber. About 0.5 cc of aqueous humor was removed and an equal volume of virus suspension was injected. Eyes were removed at 24-hour intervals up to 4 days. Microscopic study of sections showed that all 10 eyes contained cells bearing inclusion bodies.

At 24 hours the inclusion bodies were few and were identified only in cells in the region of the pectinate ligament. At 48 hours inclusion bodies were common both in the angle of the anterior chamber and in endothelial cells lining the inner surface of the cornea. The number of inclusion bodies subsequently increased and cells containing these structures were abundant in eyes removed on the fourth day. At this time the cornea was grossly opaque and of a light blue-gray color. In view of the fact that every injected eye showed conclusive evidence of infection, this method of detecting virus was chosen for further investigation. Inoculations of the eye are now made by inserting a needle through the conjunctiva and passing it forward in the subconjunctival space and through the cornea close to its junction with the sclera. This route reduces the incidence of hemorrhage.

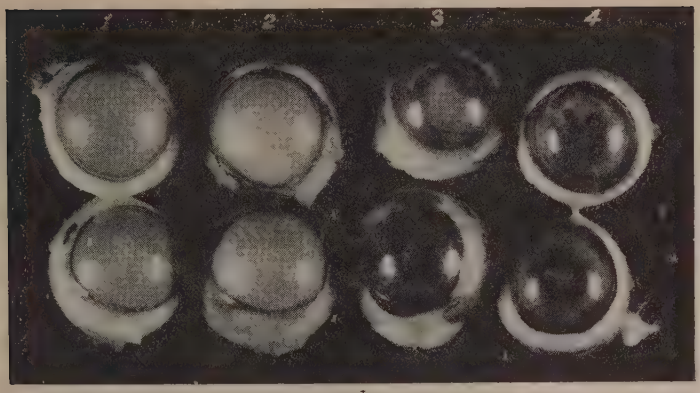

FIG. 1.

Eyes of foxes removed 5 days after intra-ocular inoculation. Inoculum in eyes 1 and 2 was active fox encephalitis virus. In eyes 3 and 4 an equal amount of virus that had been inactivated by incubation with hyperimmune serum was injected. The opacity in eyes 1 and 2 is due only to changes in the cornea. The aqueous humor remains clear. There is very little conjunctival exudate. The fibrin clot present in the anterior chambers in eyes 3 and 4 and dimly visible in some of the other eyes is a nonspecific reaction.

Several years ago we devised a technic for demonstrating quickly the inclusion bodies of canine distemper in animals that had died of that disease, by staining smears of bladder epithelium.[2] This technic has now been adapted to the demonstration of inclusion bodies in certain tissues of the eye. In fox encephalitis we ordinarily use the endothelium of the cornea and stain the smear with a modified Shorr's stain.[3] Other stains are also satisfactory and other tissues of the eye can be studied by this method. All eyes that have shown gross evidence of fox encephalitis have been found positive when suitable smears of the corneal endothelium were examined. (See Fig. 2.)

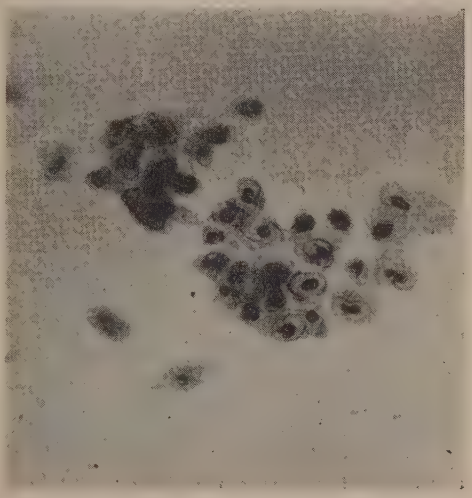

FIG. 2.

Intranuclear inclusion bodies of fox encephalitis in a smear of corneal endothelium stained by a modification of Shorr's stain.

Titrations of fox encephalitis virus have been performed by the use of eye inoculation. Three different lots of brain virus (Lot 77, Lot 93, and a recently isolated strain of virus) were used. Serial, ten-fold dilutions were prepared in Ringer's solution and the virus was injected into the anterior chamber of eyes of foxes. In each instance the virus was found fully active in a dilution of 10^{-5}. Subsequent further titration of Lot 93 showed that no infection occurred at dilutions of 10^{-6} or greater. The results of all such titrations are evident grossly, and are readily confirmed by smears at 5 days. Positive results have not been obtained at a later time in eyes that were negative at 5 days.

The smallest dose of virus that will cause an infection in the eye that is demonstrable

[2] Green, R. G., and Evans, C. A., *Cornell Vet.*, 1939, **29**, 35.

[3] Page, W. G., and Green, R. G., *Cornell Vet.*, 1942, **32**, 265.

by microscopic study of sections and smears is sufficient to cause the typical opacity of the cornea and not uncommonly will result in death of the animal.

Similar titrations have been made to determine the potency of antiserums. A specimen of serum from a group of 5 foxes that had been given a long series of hyperimmunizing injections of virus was found to neutralize completely an equal volume of a 10^{-1} dilution of virus. (See Fig. 1.)

When virus is injected into one eye, it does not produce visible infection in the other eye. Even if the second eye is traumatized by the injection of some noninfectious preparation, there has been no crossing-over of the infection in our experience. However, it is possible that in the future such cross-infections will be observed occasionally.

Summary. The difficulty involved in the study of fox encephalitis because of the highly variable susceptibility of experimental animals to fatal infection has been overcome by the use of methods that produce demonstrable, although not always fatal, infection. Instillation of the virus in the anterior chamber of the eye leads to infection of the eye while intramuscular or subcutaneous injection leads to infection in the regional lymph nodes. Extensive studies on the intra-ocular method have revealed that it is so uniformly sensitive that accurate virus titrations and neutralization tests can be made. A positive result is manifested by the development of opacity in the cornea, which can be observed grossly and is readily confirmed by the simple technic of demonstrating characteristic inclusion bodies in smears prepared with scrapings from the inner surface of the cornea.

14242

Susceptibility of the Raccoon to Fox Encephalitis.

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The virus of fox encephalitis has been shown to be characterized by a limited zoologic adaptation. In the past, only members of the family *Canidae* have been found susceptible.[1]

In a series of 5 experiments carried on over a period of 4 years, we used a total of 28 raccoons in attempts to transmit fox encephalitis to animals of this species. Only one raccoon died and inasmuch as no inclusion bodies were found on microscopic study, the results of all experiments were considered negative. Reëxamination of the sections from this animal has recently shown that inclusion bodies were present in one area of meningitis. Using the technic of intra-ocular injection,[2,3] we have now succeeded in infecting raccoons regularly. The earlier attempts

TABLE I.

Experiment No.	No. of raccoons	Dosage cc	Dosage %*	Site of inoculation	Results
209T	5	1.5	10	Intracerebral	1 died 5 days after injection
209Ta	10	1.0	5	Subcutaneous	Remained well
209Tb	4	2.0	10	Cisterna magna	" "
		2.0	10	Intramuscular	
471	9	0.5	10	Intracerebral	" "

* Percentage of tissue in the inoculum.

1 Green, R. G., Ziegler, N. R., Carlson, W. E., Shillinger, J. E., Tyler, S. H., and Dewey, E. T., *Am. J. Hyg.*, 1934, **19**, 343.

2 Evans, C. A., Yanamura, H. Y., and Green, R. G., *Science*, in press.

3 Evans, C. A., Yanamura, H. Y., and Green, R. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 183.

to infect raccoons were conducted as shown in Table I. The inoculum in each instance was a suspension of brain from foxes that died of fox encephalitis.

Upon development of the technic of intra-ocular injection as a sensitive test for the fox encephalitis virus, it was decided to reëxplore the range of zoologic adaptation of this virus.

Five normal adult raccoons were inoculated with fox encephalitis virus in the anterior chamber of the left eye (0.2 cc of a 1% pooled brain suspension, Lots 77 and 93, plus a recently isolated strain). At 48 hours, the first evidence of infection was visible as a clouding of the cornea.

At 72 hours, an inoculated eye was removed from one of the raccoons. Aqueous humor was inoculated into veal infusion broth and remained sterile. A smear of the corneal endothelium showed numerous typical inclusion bodies. The inoculated eyes of the other 4 raccoons all developed complete opacity of the cornea (Fig. 1). Two were removed on the fifth day and 2 on the sixth day. All showed numerous characteristic intranuclear inclusion bodies in the corneal smear. Culture of the aqueous humor in each instance was negative. On the fifth and sixth days, one raccoon showed hyperirritability and had convulsions, typical symptoms of fox encephalitis. Retransmission was made of virus from this animal. Virus was shown to be present in the spleen by inoculation of the eyes of 2 raccoons, both of which were grossly and microscopically positive. Further transmission from the eye of one of these animals to the eye of another raccoon was also successful.

FIG. 1.

Appearance of the eye of a raccoon 6 days after inoculation of fox encephalitis virus into the anterior chamber. The uninoculated eye remains normal.

Two of the raccoons in the original group of 5 inoculated on March 10 were subjected to surgical removal of the inoculated left eye at 3 and 6 days respectively. Both eyes were found to contain typical inclusion bodies of encephalitis. Thirty-six days after inoculation, these animals were given a second injection of encephalitis virus as a test for immunity. Two-tenths of a cc of a 10^{-4} dilution of virus (pooled fox brain, Lot 93) was injected into the anterior chamber of the right eye. This dose constitutes about 10 minimal infective doses for the eye of a fox. Both animals were killed 5 days later. At this time the cornea was clear and no inclusions could be found in smears of the corneal endothelium.

Conclusion. Although raccoons are relatively resistant to fox encephalitis, they are susceptible to invasion by the virus, as shown by a positive intra-ocular test, by demonstration of the virus in the spleen of 1 animal showing typical symptoms, and by the development of immunity as evidenced by negative intra-ocular tests 36 days after the original inoculation.

14243

Electrophoresis and Antibody Nitrogen Determinations of a Cold Hemagglutinin.*

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We have recently reported a case of gangrene of the tips of the fingers and toes in a patient whose blood contained extremely potent cold hemagglutinins.[1] During the investigation of this case, studies relative to electrophoresis and cold hemagglutinin antibody nitrogen determinations were carried out. They are reported because such details have not been published hitherto.

That cold hemagglutinins are in the globulin fraction of plasma proteins was suggested by Landsteiner[2] in his careful study of this antibody. Clough and Richter[3] and Koepplin,[4,5] using salt precipitation procedures, came to similar conclusions.

Methods and Results. 1. Electrophoresis. 50 ml of venous blood were clotted firmly for several hours at 37°C. Using aseptic technic, the serum was separated from the clot and divided into equal portions. One was stored at 4°C. The other portion was subjected to 6 absorptions at 4°C using homologous "erythrocyte ghosts."†

The agglutinated erythrocyte ghosts were separated from the serum by centrifugation at 4°-8°C. By this method the serum agglutinins were partially removed. The cold hemagglutinin titer of the unabsorbed serum was 1/2560 at 4°C; whereas the titer of the absorbed serum was 1/320 at 4°C. Further exhaustion of the absorbed serum was not attempted because of the prolonged centrifugation which would have been required to separate the weakly agglutinated ghosts from the serum. The multiple absorptions had not altered the color of the serum. (The absorptions were carried out under aseptic conditions).

The two samples of serum were then diluted separately, with diethylbarbituric acid-sodium hydroxide buffer of pH 8.63 and ionic concentration 0.10, to reduce their protein content to 1.5 g per 100 ml. Each sample was then placed in a cellophane bag and dialyzed against the buffer at 4°C for 3 days until salt equilibrium had taken place. (In this part of the experiment asepsis was not maintained).

At the conclusion of the dialysis, electrophoretic patterns of each serum were made and are reproduced in Fig. 1 and 2.

The electrophoretic curves were obtained by the method described by Longsworth, Shedlovsky and MacInnes.[6] Planimetric measurements are indicated beneath each of the figures. There is a 16% decrease in gamma globulin concentration after absorption, the areas of the other components maintaining the same ratio to the area of the albumin component. We interpret this to mean that a portion of the gamma globulin

* These studies received support from the Littauer Pneumonia Research Fund of New York University College of Medicine, the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

1 Stats, D., and Bullowa, J. G. M., *Arch. Int. Med.*, to be published.

2 Landsteiner, K., *Muenchen. med. Wchnschr.*, 1903, **50**, 1812.

3 Clough, M. C., and Richter, I. M., *Bull. Johns Hopkins Hosp.*, 1918, **29**, 86.

4 Koepplin, F., *Z. f. klin. Med.*, 1936, **129**, 512.

5 Koepplin, F., *Z. f. klin. Med.*, 1936, **130**, 784.

† Erythrocyte ghosts were prepared by hemolyzing packed saline-washed red blood cells with 10 volumes of distilled water. 18% sodium chloride solution was added to raise the tonicity to 0.9% and the hemolyzed cells were then centrifuged. After decanting the supernatant hemoglobin solution, the procedure of hemolysis by water and removal of hemoglobin was repeated 7 or 8 times to secure the ghosts almost free of hemoglobin. Before use in absorption tests, they were washed 4 times with 0.85% sodium chloride solution.

6 Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

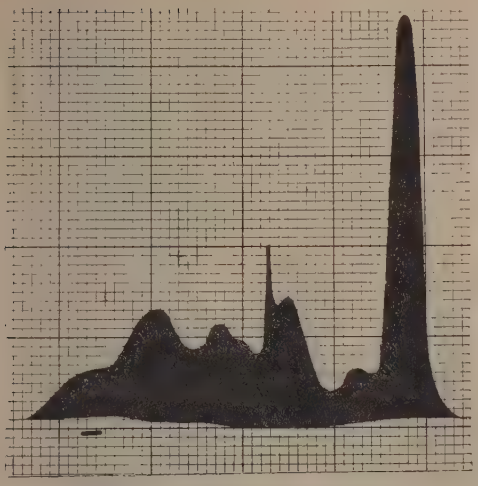

FIGURE 1. Descending electrophoretic pattern of unabsorbed serum.
Area under albumin = 170 (arbitrary planimeter units)
Area under γ globulin = 87.5 (arbitrary planimeter units)

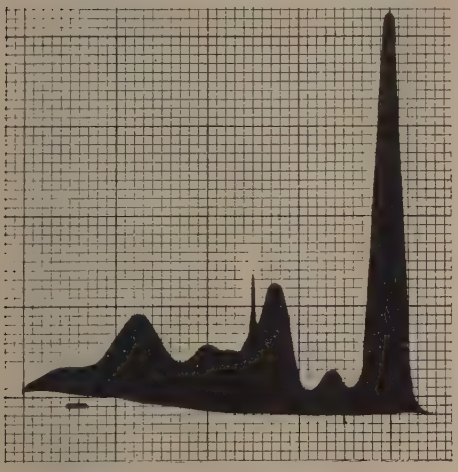

FIGURE 2. Descending electrophoretic pattern of the absorbed serum. Note: The changes in the $\alpha 1$, $\alpha 2$ and β fractions were not significant.
Area under albumin = 150 (arbitrary planimeter units)
Area under γ globulin = 64.5 (arbitrary planimeter units)

of the absorbed serum had been removed and that the cold hemagglutinins have the same mobility as gamma globulin.

2. Cold hemagglutinin antibody nitrogen. 1 ml of packed erythrocyte ghosts, almost free of hemoglobin, prepared as in the previous experiment was mixed with 5 ml of serum obtained from blood clotted at 37°C. The mixture was placed at 4°C for 6 hours. The ghosts were tightly agglutinated on the surface of the serum. After removal of the serum, 5 ml of ice cold 0.85% saline solution was added to the ghosts. The temperature was maintained at 4°C. This saline (1st washing) was replaced with 5 ml of fresh cold saline (2nd washing), etc., until the agglutinated ghosts had been washed 4 times. Throughout all of these procedures, the temperature did not rise above 6°C and was maintained as close to 4°C as possible.

The packed agglutinated washed ghosts were resuspended in 2 ml of 0.85% saline solution and placed in the water bath at 37°C. In a few minutes the agglutination broke up and the ghosts were freely dispersed. After 30 minutes, during which time the tube was shaken several times, the tube was centrifuged at room temperature. The supernatant clear saline solution was removed (resuspended antibody).

One ml of packed washed normal ghosts was mixed with 2 ml of 0.85% saline solution and kept at 4°C for one hour. The temperature was then increased to 37°C for one hour and the tube centrifuged at 22°C.

Cold hemagglutinin titrations of the original serum, the 4 washings and the resuspended antibody were performed according to the technic previously described.[1] Total nitrogen determinations of the washings, resuspended antibody, saline solution and saline solution which had been in contact with normal ghosts were performed by a micro-Kjeldahl technic.

Chart I summarizes these results.

CHART I.

Specimen	Titer cold hemagglutinins	Total nitrogen mg/ml
1st washing	1/40	0.614, 0.722
2nd "	1/20	0.153, 0.146
3rd "	1/10	0.059, 0.060
4th "	1/10	0.035, 0.028
Resuspended antibody	1/2560	1.483, 1.464
Original serum	1/5120	
Saline solution	—	0.009, 0.003
Saline solution in contact with normal ghosts	—	0.004, 0.004
Blank for reagents (Blank has been deducted)	—	0.027, 0.034

The high nitrogen values for the washings are due to non-antibody protein of the serum. The progressive reduction in the values indicates the removal of this protein by successive dilution. The possibility that non-antibody protein was non-specifically taken up in the lattice of agglutinated ghosts cannot be excluded. Therefore a portion of the nitrogen in the resuspended antibody may not represent antibody nitrogen.

Summary. Study of a human cold hemagglutinin revealed:

1. The cold hemagglutinin had the electrophoretic mobility of gamma globulin. 2. A cold hemagglutinin titer of 1/2560 at 4°C was equivalent to 1.473 mg per ml of antibody nitrogen.

14244

Metabolism of Sulfapyridine in the Dog.

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The therapeutic efficacy of the sulfonamides has stimulated an interest in the possible changes these compounds may undergo in the animal body. It is well known that the sulfonamides are acetylated in the human body and in this form they are relatively inactive. However, it is possible that other forms of conjugation or alteration in structure may occur which might modify their toxicity or activity.

Scudi[1] reported the isolation from dog urine of an hydroxysulfapyridine conjugated with glucuronic acid following the administration of sulfapyridine. No details were given on the isolation of this compound nor data concerning the probable position of the hydroxy group. Thorpe, Williams, and Shelswell[2] have suggested the possibility that the hydroxyl group might be introduced in the animal body in either the 2 or 3 position of the benzene ring of the sulfonamide.

Our interest in this problem was aroused when we were unable to isolate from the urine sulfapyridine as the reduction product of 2-(p-nitrobenzenesulfonamido) pyridine when the latter compound was administered orally to dogs. The same difficulty was encountered when sulfapyridine was administered. Sulfapyridine added to urine can be isolated readily by prolonged ether extraction. We therefore, decided to attempt the isolation of the diazo reacting substances which are excreted in dog urine when sulfapyridine is administered orally. The values given for diazo reacting substances in the urine are those determined by the Bratton and Marshall[3] method, using sulfapyridine as a standard.

Four dogs were given orally 5 g of sulfapyridine daily for 4 days and the urines were collected daily for 6 days, and preserved with chloroform. The combined urines gave a total sulfapyridine value of 67 g and no increase in color was obtained after acid hydrolysis indicative of the fact that the dog apparently does not conjugate any of the sulfapyridine as an acetyl derivative. Prolonged ether extraction in a continuous extractor for 9 hr resulted in the extraction of 9 g of diazotizable material as determined colorimetrically. When small quantities were extracted with ether in a more efficient extractor, 16.5% of the total diazotizable material was extracted by ether and 13.8% was extracted in the first 3 hours. This value of 16.5% probably represents the total amount of sulfapyridine which is excreted unconjugated. The gummy material extracted with

1 Scudi, J. V. *Science*, 1940, **91**, 486.

2 Thorpe, W. V., Williams, R. T., and Shelswell, *Biochem. J.*, 1941, **35**, 52.

3 Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

ether yielded a total of 2 g of crystalline material which melted at 190-191°C and no depression of the melting point was produced when mixed with sulfapyridine. The nitrogen content (Kjeldahl) was 16.5% (calculated for sulfapyridine 16.8%).

The urine extracted with ether was treated with an excess of lead acetate and the mixture acidified with acetic acid. The heavy precipitate obtained was filtered off. The filtrate was made alkaline with sodium hydroxide (10%) and the precipitate of lead hydroxide obtained, was filtered off and the filtrate discarded. The lead precipitate was suspended in water and the lead removed with hydrogen sulfide. The filtrate from the lead sulfide containing 45 g of diazotizable material or 67% of that present in the original urine. A portion of this filtrate containing the equivalent of 9 g sulfapyridine was treated with silver nitrate which produced a heavy flocculent precipitate. This precipitate was filtered off and resuspended in boiling water which dissolved the greater part and from which it would again precipitate on cooling. This precipitate was collected and dried *in vacuo*. On analysis it showed a silver content of 29.6%, nitrogen content of 6.45% and a color value equivalent to 35.2% sulfapyridine. On hydrolysis with N HCl the solution reduced Benedict's solution and the glucose equivalent was 40.1%. This compound is probably the silver salt of a glycuronic acid conjugate of a sulfapyridine derivative, which has been described by Scudi,[1] although we were never able to isolate a crystalline product or one that gave a constant silver value, the silver contents varying from 26.7% to 29.5%.

The remainder of the lead free filtrate was evaporated to 1000 cc and 100 g sodium hydroxide was added and mixture refluxed for 5 hours. This hydrolyzed solution was neutralized to approximately pH 6.5 and extracted with ether in a continuous extractor for 24 hours. During the ether extraction a crystalline product separated from the ether. A total of 26.2 g of crystalline material was obtained. This was dissolved in acetone by addition of water. The material was light pink and on drying *in vacuo* lost its crystalline appearance and approximately 10% in weight. It gave a positive diazo reaction and gave a color equivalent to sulfapyridine of 89%. Ferric chloride gave a deep purple color which changed rapidly to dark brown. It melted at 190°C and a depression of the melting point was produced when mixed with sulfapyridine. The nitrogen content was 15.6%.

14 g of the above compound was refluxed with 100 cc 10% hydrochloric acid for three hours. The material went completely in solution but after 2 hours of boiling a crystalline material began to precipitate. The mixture was cooled overnight and the crystalline material separated and dried. After recrystallization from water 5.2 g of crystalline material was obtained. This material gave a positive diazo reaction and gave a color value equivalent to that given by sulfanilic acid. The $FeCl_3$ test was negative. The M.P. was above 300°C. The nitrogen content (Kjeldahl) was 7.91%, calculated for sulfanilic acid 8.09%.

The acid filtrate from the above crystals was treated with an excess of picric acid. The copious precipitate was filtered off and dried. Recrystallization from water gave prism-shaped crystals which melted at 212° and the melting point was depressed when mixed with 2-amino-pyridine picrate. The picrate was suspended in water acidified with HCl and the picric acid removed with ether. The extracted solution was evaporated to dryness and the residue readily dissolved in 15 cc absolute alcohol. The cautious addition of ether resulted in the appearance of crystals and then 10 volumes of ether was added. On recrystallization, 2.85 g of crystalline material was obtained. It gave a strong positive $FeCl_3$ test, negative diazo test, and melted at 126°C. Analysis: Cl-23.7%; Nitrogen (Kjeldahl) 18.7%. Calculated for 2-amino-hydroxy-pyridine hydrochloride—Cl 23.5%, nitrogen 19.11%. Our data indicate that sulfapyridine is excreted in dog urine largely combined with a reducing substance presumably glucuronic acid. Alkaline hydrolysis of this compound results in the liberation of a compound giving a positive ferric chloride test indicative of the presence of an hydroxyl group. Acid hydrolysis of this compound permitted the isolation of sulfanilic acid and a pyridine residue which gives a positive

ferric chloride test. Therefore the dog apparently metabolizes sulfapyridine in greater part by introducing an hydroxyl group in the pyridine ring and then conjugating with a carbohydrate. This hydroxy derivative possesses some therapeutic effect in mice injected with hemolytic streptococcus and produces bacteriostasis *in vitro*, but the efficacy is less than that of sulfapyridine. As much as 80% of the diazo reacting compounds appearing in dog urine following the ingestion of sulfapyridine is in the form of this glycuronide. Only a small portion which probably is not greater than 16 percent is excreted as sulfapyridine. At least in the dog no hydroxyl group is introduced in the benzene ring and this is further verified by the fact that sulfanilamide is excreted without change.

Summary. The ingestion of sulfapyridine in the dog is followed by the appearance in the urine of a glycuronide of a hydroxy derivative of sulfapyridine. The hydroxy group is attached to the pyridine ring.

14245 P

Enhancement with Phenol of the Serological Reactivity of Lymphogranuloma Venereum Antigens.

CLARA NIGG AND BETTY M. BOWSER. (Introduced by W. E. Bunney.)

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Three types of complement-fixing antigen prepared from yolk sac cultures of the agent of lymphogranuloma venereum have been described; *viz.*, (1) resuspended high speed sediment,[1] (2) soluble antigen in high speed supernates,[2] (3) whole suspensions inactivated with urea or ether.[3] The third type was approximately four times as active as high speed sediment.

Attempts by one of the authors (Nigg) to find a satisfactory preservative for lymphogranuloma venereum complement-fixing antigens, showed that phenol enhanced the activity of the antigen specifically since such phenolized antigens did not react with normal sera. This observation was studied further and is the subject of this note.

Table I shows the complement-fixing activity of a 10% yolk sac suspension treated with (a) 2% urea, (b) 0.25% phenol and (c) 0.5% phenol. Preparations were stored at icebox temperatures. Two separate fractions of suspension 56 when treated with 0.25% phenol failed to show increased activity when tested 8 to 30 days later. Two fractions treated with 0.5% phenol were 4 and 8 times respectively as active as the urea-treated antigen.

While 0.25% phenol had little enhancing effect at icebox temperatures, Table II shows approximately a 4-fold increase in activity when these suspensions were subjected to the following temperatures: (a) 37°C for 6 weeks (not tested earlier), (b) 56°C for 48 hours, and (c) boiling water for 10 minutes. Furthermore, the centrifuged (2,000 rpm for 10 minutes) supernates of the heated suspensions were almost as active as the whole heated suspensions. This represents a considerable purification since the slightly opalescent supernates, after removal of tissue debris and coagulated protein, were almost as active as the whole heated turbid suspensions.

That the phenol enhancement is apparently specific is shown in diagnostic tests (Table III) with positive and negative sera, comparing 2 phenolized antigens with urea-treated antigen. Antigen 57-H was treated

[1] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[2] Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 300.

[3] Nigg, C., PROC. SOC. EXP. BIOL. AND MED., **1942, 49, 132.**

TABLE I.
Effect of Phenol on the Complement-fixing Antigen of Lymphogranuloma Venereum.

Antigens: 10% suspensions treated with:		L.V. serum pool 12 (1-60) Antigen dilutions: 1-200	1-400	1-800	1-1600	1-3200	1-6400	Interval between preparation and testing, days
2% urea:	56-1	4+	4+w	1+	0			
0.25% phenol:	56-2	4+	4+	1-2+	0			30
	56-5	4+	4+	3-4+	tr			8
0.5% phenol	56-7B	4+	4+	4+	4+			8
	"	4+	4+	4+	4+	2+	0	37
	56-8	4+	4+	4+	4+	2+		7
	"	4+	4+	4+	4+	4+	2+	28

Results are stated in terms of the degree of fixation.

TABLE II.
Effect of Temperature on Phenolized Complement-fixing Antigens of Lymphogranuloma Venereum.

Antigens: 10% suspensions treated with:	Temperature exposure		L.V. serum pool 12 (1-60) Antigen dilutions: 1-200	1-400	1-800	1-1600	1-3200	1-6400
2% urea:								
56-1	Icebox 8 wk		4+	4+	1+	0		
0.25% phenol:								
56-2	Icebox 6 wk		4+	4+	3+w	0		
"	Room temperature 6 wk		4+	4+	3+w	0		
"	37°C 6 wk		4+	4+	4+	4+	1-2+	
2% urea:								
57-1	Icebox 5 wk		4+	4+w	0			
0.25% phenol:								
57-F-5a	56°C 48 hr	Whole susp	4+	4+	4+	3-4+	1-2+	0
		Supernate	4+	4+	4+w	1+	0	0
57-F-5b	Boiled 10 min.	Whole susp.	4+	4+	4+	4+	1-2+	0
		Supernate	4+	4+	4+	4+w	1+	0

with 0.25% phenol at 56°C for 18 hours. Antigen 57-F-6a is the slightly opalescent supernate from a boiled suspension likewise containing 0.25% phenol. These 3 antigens were prepared from the same initial 10% yolk sac suspension. One unit of each antigen, determined by preliminary titration, was used. The enhancement is apparently specific since suspensions of normal yolk sac treated in identical manner do not give fixation even in a 1-250 dilution. The anticomplementary activity of all antigens and sera was ruled out by appropriate controls.

Discussion. The enhanced complement-fixing activity of phenolized suspensions is of considerable practical significance; *viz.*, (1) the activity is increased from 4 to 8 times, (2) objectionable turbidity is removed, and (3) phenol acts as an effective preservative without the deleterious effect produced by formalin, *e.g.*,[3] and (4) thermostability facilitates shipping these antigens without refrigeration and their use in warm climates.

Information has also been obtained regarding the nature of the complement-fixing substance, *viz.*, (1) its stability at boiling temperature, (2) its activation by phenol and (3) its properties which are not characteristic of native proteins. Studies on the chemical nature of the active substance are in progress.

In a limited number of tests with sera from early syphilis non-specific fixation was never encountered with the phenolized antigens, whereas it was frequently observed with both the resuspended high speed sediment[4] and urea-inactivated antigens.[5]

[4] Shaffer, M. F., Rake, G., Grace, A. W., McKee, C. M., and Jones, H. P., *Am. J. Syph., Gon., and Ven. Dis.*, 1941, **25**, 699.

[5] Nigg, C., unpublished data.

TABLE III.
Comparison of Phenolized Antigens with Urea Antigen in Diagnostic Tests for Lymphogranuloma venereum.

Sera	Antigen 57-H treated with 0.25% phenol at 56°C 18 hr (1-1000) Serum dilutions: 1-5	1-10	1-20	1-40	1-80	1-160	Control antigen 11-C treated with 0.25% phenol at 56°C 18 hr (1-1000) *
13			4+	4+	4+	2+	0
Re			4+	4+	4+	2-3+	0
Ga	4+w	2-3+	0				0
BB	0						0
	Supernate of antigen 57-F-6a treated with 0.25% phenol, boiled 10 min (1-1000)						Supernate of control antigen 11-B treated with 0.25% phenol, boiled 10 min (1-1000)
13			4+	4+	4+	3-4+	0
Re			4+	4+	4+	3-4+	0
Ga	3-4+	2+	0				0
BB	0						0
	Antigen 57-1 treated with 2% urea (1-200)						Control antigen 9-10 treated with 2% urea (1-200)
13			4+	4+	4+w	3-4+	0
Re			4+	4+	4+	3-4+	0
Ga	3-4+	2+	0				0
BB	0						0

* The lowest dilution of serum which was used with the specific antigen was used in testing the control antigens which were prepared from normal yolk sacs.

The purified phenolized antigens may possibly eliminate, to a certain extent at least, the cross reactions which preclude differential serological diagnosis in the lymphogranuloma-psittacosis group.[6-10] Similar enhancement with phenol might obtain with antigens of other members of this group.

The identity of the skin-reactive and complement-fixing antigens is being investigated in coöperation with Dr. A. W. Grace, by comparing in Frei tests the purified phenolized antigens with other yolk sac antigens.

The exact conditions which produce phenol enhancement are not yet thoroughly understood. Occasional preparations have been encountered in which enhancement was comparatively slight with the same concentration of phenol which regularly gave marked enhancement of other preparations. That the pH influences the degree of enhancement is indicated by preliminary experiments.

6 Rake, G., Eaton, M. D., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 528.

7 Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

8 Eddie, B., and Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 291.

9 Rake, G., Jones, H. P., and Nigg, C., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 449.

10 Levine, S., Holder, E. C., and Bullowa, J. G. M., *J. Immunol.*, 1943, **46**, 183.

14246 P

Chromatin Structures Suggesting a Nuclear Apparatus in the Large Bodies of *B. funduliformis.**†

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Ledingham[1] indicated the presence of "nucleus like" chromatin bodies in the large swollen forms of the organism of bovine pleuropneumonia. Klieneberger and Smiles[2] and Klieneberger[3] studying the mode of reproduction in the pleuropneumonia group observed that the chromatin divides and is distributed into the new cells. This chromatin gives the Fuelgen reaction indicating the presence of nucleic acid and Klieneberger attributes nuclear character to this material.

We have seen exactly similar chromatin material in the large bodies of various bacterial species.[4] We have been able to study it best in 3 strains of *Bacteroides funduliformis* because of the regularity with which these strains produce large bodies[5] and because of the excellent staining of their chromatin material. Pleuropneumonia-like (L) colonies frequently appeared among the ordinary bacterial colonies of 2 of these strains.

The present report deals with studies made on 1 of these strains which we have designated as Strain No. 132. This strain has been carried through more than 50 subcultures and continues to produce L variants in abundance. These L variants have been isolated in pure culture. They did not revert to their parent bacterial form throughout 30 subcultures on agar. They were strictly anaerobic, just as their parent bacterial forms.

The chromatin in the bacilli and in the L forms is best seen in preparations made by a modification of Klieneberger's agar fixation technic.[2] Agar blocks bearing the organisms are inverted onto cover slips and fixed for several days with Bouin's solution. The agar is then peeled away and the cover slip rinsed, stained with Giemsa, differentiated with ascitic fluid and mounted in balsam. The cover slip should never be allowed to dry until just before mounting, as shrinkage makes it impossible to see the precise distribution of chromatin within the organisms.

In broth, Strain 132 grows in the form of regular bacilli which swell up after 9 to 20 hours into large round bodies. The further development of these large bodies is best followed by transferring them to ascitic agar plates on which they produce either a bacterial or a pleuropneumonia-like colony. In a previous note[5] we described the fractionation of some of the large bodies into bacilli or the extrusion of multiple filaments which then segmented into bacilli. From other large bodies, small granules were observed to grow out and these developed into pleuropneumonia-like colonies.

With more experience in staining, it was seen that the large bodies contain deeply stained chromatin material embedded in a lightly stained material which may be most conveniently designated as cytoplasm. As illustrated in the photographs, the chromatin may be present in the form of discrete granules, in thread-like branching masses or in a single dense mass. When the large bodies reproduce ordinary bacilli, it was seen that they extrude tongues of "cytoplasm" and the chromatin grows out into these cytoplasmic filaments, which then segment to yield the

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

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1 Ledingham, J. C. G., *J. Path. Bact.*, 1933, **37**, 393.

2 Klieneberger, E., and Smiles, J., *J. Hygiene*, 1942, **42**, 110.

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4 Dienes, L., *J. Bact.*, 1942, **44**, 37.

5 Dienes, L., and Smith, W. E., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 297.

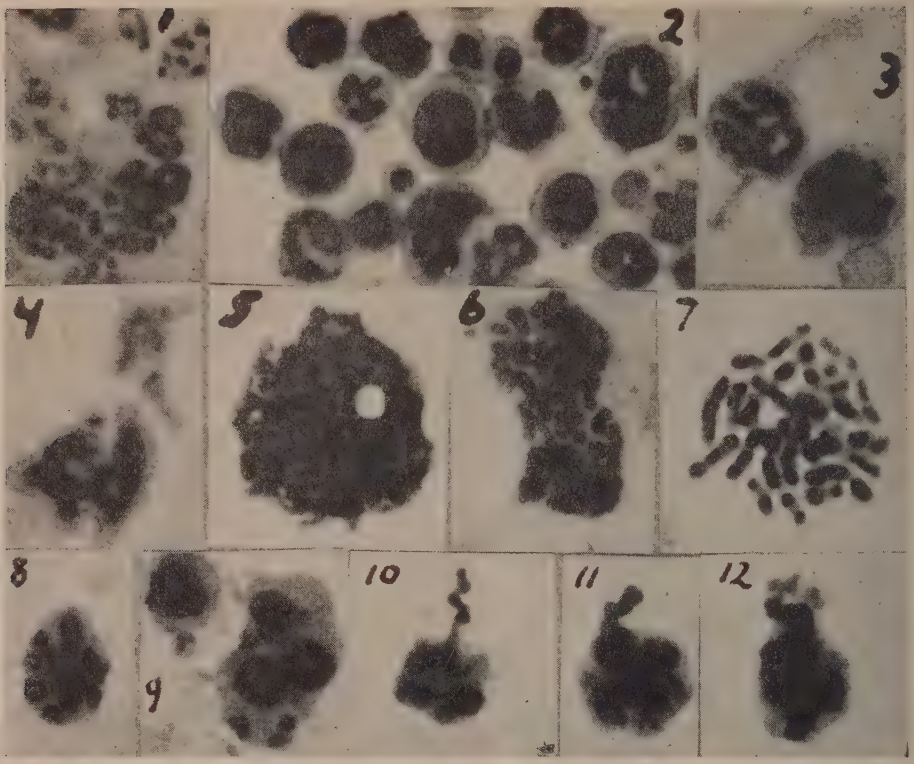

FIG. 1.

All photographs were made from Giemsa-stained preparations with high magnification (3000 ×). 1. Large bodies in a 24-hr broth culture. The chromatin is present in discrete granules. 2. Same culture as 1 after 6 hrs incubation on ascitic agar. The chromatin is in bands or condensed to compact masses. 3. Same preparation as 2. The chromatin is present in bands. The indentation in one of the large bodies indicates its development from two bacteria. See following note. 4. Same preparation as 2. The large body is deformed and begins to extrude bacterial filaments into which the chromatin is growing. 5 and 6. Fractionation of the chromatin before the large bodies disintegrate into bacteria. The large bodies are increased in size. 6½ hrs incubation on ascitic agar. 7. Same preparation as 5 and 6. Bacteria produced by disintegration of the large body. Every bacterium has one or more chromatin granules. 8 and 9. Large bodies with chromatin condensed into a few large masses. 3 hrs incubation on ascitic agar. 10, 11, 12. Growth of pleuropneumonia-like colony starting from the large chromatin granules. 3 hrs incubation on ascitic agar.

bacilli. The development of bacilli is often preceded by the breaking up of the chromatin into granules which are distributed into the bacteria. Frequently, the "cytoplasm" fractions into masses around the chromatin granules and bacilli are thus formed within the large body. The chromatin is present in the bacilli as small discrete granules or elongated masses. When the large bodies reproduce pleuropneumonia-like colonies, there is no extrusion of cytoplasmic filaments and the chromatin is seen in 1 or several large masses. These masses extrude fine chromatin filaments which become nodular and give rise to the pleuropneumonia-like growth.

These observations support the view that the chromatin has a nuclear character and that it is connected both with the reproduction of bacteria and of the L type of growth.

14247

Elimination of Ulcerative Cecitis from a Rat Colony by Chemotherapy of Mothers.

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In a previous paper[1] we showed that administration of sulfaguanidine to the mothers during pregnancy and lactation largely prevented the development of ulcerative cecitis in the young of rats. The experiments were carried on under special controlled conditions, but their real object was to devise a method whereby, in practice, cecitis could be eliminated from a rat colony in which the disease had been highly prevalent for several years. The present report describes the outcome of such a practical trial.

Ulcerative cecitis, thoroughly described in the recent literature,[2] "broke out" in the Stanford rat colony in 1940. Young rats were not affected but from the age of 6 months on from 50 to 80% of the animals showed the disease, usually in an advanced stage. It was impossible to continue the regular work of the laboratory. Between September 1941 and June 1942 a large number of stock animals were killed to determine the actual incidence of cecitis. It should be emphasized that during this entire period there was no evidence of a spontaneous decrease of the disease. The results of this survey are shown in Table I. From February 1942 on, all the females used for breeding received 0.5% sulfaguanidine in their stock ration during pregnancy and lactation. This procedure is still being continued at the present time (May 1943). Between January and May 1943 stock rats born of the mothers treated with sulfaguanidine, but having themselves received none of the drug, were sacrificed in order to compare them with the controls from untreated mothers. The results are also shown in Table I.

It is seen that in rats from 4 to 8 months old the over all incidence of cecitis was reduced from 55% to 4.5%. Furthermore the few animals from treated mothers which developed cecitis showed the disease only in mild form.

Conclusions. We have previously shown that ulcerative cecitis of rats can be prevented by adding sulfaguanidine to the food ration. The present observations indicate that the disease may be largely eliminated from a colony in which it is endemic by treatment of the mothers during pregnancy and lactation only. This procedure effects an economy of chemotherapy which reduces the expense to a reasonable level. These observations confirm and amplify those previously reported.

TABLE I.

Reduction of Incidence of Ulcerative Cecitis in a Rat Colony after Treating Mothers with Sulfaguanidine.

Age when sacrificed (mo)	2-3	3-4	4-5	5-6	6-7	7-8
	Controls from Untreated Mothers.					
No. of animals	24	27	50	57	52	75
No. with cecitis	1 (4.2%)	2 (7.5%)	22 (44%)	31 (54%)	26 (50%)	50 (66%)
	Animals from Mothers Who Received Sulfaguanidine.					
No. of animals			32	45	22	33
No. with cecitis			1 (3%)	3 (6.5%)	0 (0.0%)	2 (6.0%)

All rats 4-8 months old from untreated mothers 234, incidence of cecitis 55.0%.
All rats 4-8 months old from treated mothers 132, incidence of cecitis 4.5%.

1 Bloomfield, A. L., and Lew, W., *Am. J. Med. Sc.*, 1943, **205**, 383.

2 Stewart, H. L., and Jones, B. F., *Arch. Path.*, 1941, **31**, 37.

14248

Action of Strophanthidin-3-propionate, -butyrate, and -benzoate.

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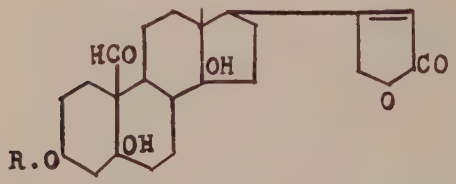
The secondary hydroxy group on carbon atom 3 of cardiac aglycones is chemically very reactive. By the interaction of a molecule of sugar with the aglycone at this position, synthetic glycosides of strophanthidin, digitoxigenin, digoxigenin, and periplogenin were successfully prepared at the Department of Chemistry, Columbia University under the direction of Professor Robert C. Elderfield.[1,2] It has been shown[3,4] that these synthetic glycosides are more potent than their parent glycosides, namely, cymarin, digitoxin, digoxin, and periplocymarin, respectively. Esters of strophanthidin can be also conveniently synthesized.[5] The present study consisted of the preparation of 3 such compounds, and the determination of their potency in cats. They are, chemically, strophanthidin-3-*n*-propionate, -*n*-butyrate, and -benzoate, and conform to the following structure:



wherein R denotes $CH_3 \cdot CH_2 \cdot CO$, $CH_3 \cdot (CH_2)_2 \cdot CO$, or $C_6H_5 \cdot CO$.

The aglycone strophanthin was isolated from the seeds of *Strophanthus kombé* according to the method of Jacobs and Heidelberger.[6] We are much indebted to Professor Robert C. Elderfield for his generosity in giving us his modification for the improvement of the yield of this aglycone. The propionate and the butyrate were obtained by Neumann's procedure,[5] and the benzoate by that of Windaus and Hermanns.[7] In principle, strophanthidin was allowed to react with the acid chloride in the presence of pyridine. The esters were all purified by repeated recrystallization from methanol. The propionate melted at 234.5-235°C, the butyrate at 212-213°C, and the benzoate at 225-226°C (corrected), the rate of heating being moderate. The results of combustion analyses satisfied the required compositions.

For cat experiments, stock solutions of 1:1000 were made with strophanthidin-3-*n*-propionate and -*n*-butyrate, containing 47.5% ethanol by volume. Dilutions of 1:50,000 were employed for intravenous injection until death occurred in the same manner as previously reported.[8] The rate of injection was 1 cc per minute. The benzoate was much less soluble in water, requiring 71.25% ethanol by volume for a solution of 1:500. The material would crystallize out in further dilutions. It was thus necessary to inject the stock solution intravenously at the rate of 0.08 cc per minute followed by 1 cc of saline by means of a 3-way stopcock—a procedure previously adopted in this laboratory.[9]

The results are shown in Table I. Judging from the geometric mean, that is, the antilogarithm of the mean of logarithms of in-

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TABLE I.
Results in Cats.

Compound	Sex	Body wt, kg	Dose to kill, mg per kg	Mean (geometric) lethal dose ± standard error, mg per kg
Strophanthidin				0.3250 ± 0.0232*
Strophanthidin-3-acetate				0.1866 ± 0.0246†
	F	1.900	0.1726	
	M	1.878	0.3695	
	M	1.972	0.2779	
	M	1.858	0.6362	
Strophanthidin-3-*n*-propionate	M	1.904	0.2626	0.2573 ± 0.0377
	F	2.144	0.4300	
	F	2.081	0.2379	
	F	2.707	0.1101	
	F	2.117	0.2834	
	F	1.964	0.1874	
	F	1.857	0.4157	
	F	1.977	0.4982	
	F	1.861	0.4449	
	M	1.783	0.2961	
Strophanthidin-3-*n*-butyrate	M	1.694	0.4534	0.4263 ± 0.0220
	F	2.103	0.5544	
	F	2.049	0.3348	
	F	2.574	0.6267	
	F	2.169	0.4168	
	F	2.014	0.3307	
	M	2.238	2.8776	
	F	1.825	2.3233	
	M	1.806	2.5692	
	F	1.840	4.5217	
Strophanthidin-3-benzoate	F	1.937	1.4042	2.717 ± 0.2610
	F	2.348	2.7257	
	M	1.918	2.7320	
	M	2.502	2.6219	
	M	1.803	2.7510	
	M	2.068	3.7524	

* Mean of combined data published previously.[3,8]
† Previously reported.[3]

dividual doses, the propionate is more potent than strophanthin in cats; but the butyrate and the benzoate are both less active. Our data on the butyrate are thus in agreement with those of Neumann.[5] In a previous communication,[3] evidence was presented that strophanthidin-3-acetate was more powerful than strophanthidin. It is apparent that among the acyl radicals which form esters with strophanthidin, the members of the lower molecular weight, namely, acetyl and propionyl, enhance the activity of strophanthidin in cats. On the other hand, butyryl and benzoyl groups decrease its activity.

Summary. Weight for weight, strophanthidin-3-*n*-propionate is more potent than strophanthidin in cats, while strophanthidin-3-*n*-butyrate and -benzoate prove to be less active.

14249

Potency of Cymarin and Coumingine Hydrochloride as Influenced by Environmental Temperature.

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From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, and the Laboratories for Experimental Medicine, University of Cincinnati, Cincinnati.

The influence of season and environmental temperature on the susceptibility of frogs to digitalis and its allied products has been repeatedly investigated. Moschkowitsch,[1] Edmunds and Hale,[2] and Gottlieb[3] reported that summer frogs were more resistant than winter frogs. Ziegenbein[4] believed that the season of the year made little difference in the susceptibility of frogs. Dixon[5] found less than 50% variation in frogs' sensitivity to digitalis throughout the year. Mansfeld and Horn,[6] using the isolated sinus of the frog's heart, stated that the months of June, July, and August did not give as reliable results as other months for strophanthin even though the temperature at which the tests were made was comparable. A majority of other workers observed an increase in sensitivity of frogs during the summer months. Thus, Focke[7,8] emphasized that summer frogs were more reactive to digitalis. Baker[9] concluded that ouabain increased in potency fourfold from 10° to 30°C, while digitalis showed only a difference of 25%. Weizsäcker[10] demonstrated that with the isolated frog's heart, the lower the temperature, the more time required for the completion of strophanthin action. Sollmann, Mendenhall, and Stingel[11] found that the toxicity of ouabain for frogs increased markedly with temperature, the increase per degree of temperature being much greater at the lower than at the higher temperatures. Smith and McClosky[12] proved that by intravenous injection the susceptibility of the frog's heart to digitalis increased with temperature in much the same manner as its susceptibility to ouabain. Gander[13] experimented with gitalin and gitaligenin on the isolated frog's heart, and came to the conclusion that their fixation and action were accelerated by elevation of temperature.

In this laboratory, a specially air-conditioned room was constructed. It was proposed that various drugs be studied at different room temperatures. In another communication,[14] results were presented to show that certain drugs at 40°C were several times as potent as at 20°C in mice. In the present investigation, cymarin and coumingine hydrochloride, both digitalis-like products, were assayed according to the U.S.P. XI[15] 1-hour frog method. The room was adjusted to 16°, 20°, 25°, 30°, 35°, and 40°C. Several concentrations of the solutions were employed in order to avoid an excessive volume of doses. Owing to the free air flow, evaporation took place at the surface of the water bath, so that the temperature of the bath, recorded by a thermometer, was lower than

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the room temperature. The frogs (*Rana pipiens*) of the same batch were acclimatized at each temperature for 24 hours, except that at room temperature of 40°C they were injected without prolonged adaptation, since an overwhelming majority would have died at such temperature level if kept there for more than 3-4 hours. The entire work was carried out during the months of March, April, and May.

The results are summarized in Table I. The median systolic doses (SD_{50} ± standard error) were computed by the combined slope.[16] The general trend is unmistakable, namely, the higher the room or bath temperature, the more potent the drugs. With cymarin the data appear to fall into 3 groups: the frogs were least sensitive between 13° and 15.5°C, more sensitive between 20° and 29°C, and most sensitive at 33°C, bath temperature. No explanation is available as to why the frogs were more susceptible at 24.5° than at 20° and 29°C (bath temperature). With coumingine hydrochloride, the results ran a more uniform course, that is, without exception, the higher the temperature, the more potent the alkaloid as shown by the SD_{50}. Indeed, coumingine hydrochloride is approximately 5 times as potent at 33°C as at 13°C, bath temperature.

The frog is a poikilothermic animal, and its change in susceptibility to cymarin and

[16] Miller, L. C., Bliss, C. I., and Braun, H. A., *J. Am. Pharm. Assn.*, 1939, **28**, 644.

TABLE I.

Room temperature, °C	Temperature of bath, °C	Dose, μg/g	No. in systole / No. used	LD_{50} ± S.E. (by combined slope), μg/g
		Cymarin.		
		1.00	1/5	
		1.10	3/10	
16	13	1.25	1/5	1.374 ± 0.096
		1.40	1/5	
		1.60	4/5	
		1.80	3/3	
		1.00	2/10	
		1.10	4/10	
20	15.5	1.25	10/15	1.158 ± 0.061
		1.40	9/12	
		1.60	3/3	
		0.62	0/5	
		0.70	2/5	
25	20	0.80	3/5	0.816 ± 0.056
		0.90	3/5	
		1.00	3/5	
		1.10	4/4	
		0.56	0/5	
30	24.5	0.62	4/10	0.651 ± 0.043
		0.70	6/9	
		0.80	5/5	
		0.50	0/5	
		0.56	2/10	
		0.62	2/9	
35	29	0.70	5/10	0.788 ± 0.040
		0.80	4/10	
		0.90	5/10	
		1.00	3/4	
		1.10	4/4	
		0.45	2/5	
		0.50	5/10	
40	33	0.56	5/10	0.529 ± 0.032
		0.62	6/10	
		0.70	9/10	

TABLE I (Continued).

Room temperature °C	Temperature of bath, °C	Dose, μg/g	No. in systole / No. used	$LD_{50} \pm$ S.E. (by combined slope), μg/g
		Coumingine Hydrochloride.		
		3.00	3/10	
16	13	3.60	2/10	4.154 ± 0.263
		4.50	4/10	
		5.60	5/5	
		3.00	1/10	
		3.30	1/10	
20	15.5	3.60	4/11	3.868 ± 0.209
		4.00	9/13	
		4.50	3/3	
		1.80	1/10	
		2.00	7/11	
25	20	2.25	6/9	2.148 ± 0.103
		2.50	8/15	
		2.75	5/5	
		1.40	0/5	
		1.60	1/5	
30	24.5	1.80	5/10	1.961 ± 0.100
		2.00	5/10	
		2.25	7/10	
		2.50	3/4	
		1.25	1/10	
		1.40	2/10	
35	29	1.60	4/8	1.733 ± 0.095
		1.80	5/10	
		2.00	6/9	
		0.80	2/5	
40	33	0.90	2/5	0.810 ± 0.082
		1.00	5/5	
		1.10	5/5	

coumingine hydrochloride due to environmental temperature may be more apt to correspond to a chemical reaction than that of warm-blooded animals. The results of the present study, however, do not show a directly proportional relationship, for the points of temperatures versus doses (or logarithms) do not fall on a straight line.

Summary. The susceptibility of the frog's heart to cymarin and coumingine hydrochloride increases with the rise of environmental temperature. Coumingine hydrochloride is approximately 5 times as potent at 33°C as at 13°C (bath temperature), and cymarin is more than twice as active at 33°C as at 13°C.

14250 P

Effect of Environmental Stilbestrol in Shortening Prolonged Gestation in the Lactating Rat.

Charles K. Weichert. (Introduced by L. H. Schmidt.)
From the Zoölogical Laboratory, University of Cincinnati.

Estrogen is usually administered to experimental animals by injection, inunction, subcutaneous implantation of pellets or by the oral route. Little or no attention has been paid to the possible effects of estrogen that may be present in cages in which treated animals are kept and in whose excreta quantities of hormone may be eliminated. The present report shows that minute quantities of the synthetic estrogen diethylstilbestrol, by its mere presence in the animal's cage, may reduce the gestation periods of inseminated lactating rats to normal.

The length of the gestation periods of lactating rats inseminated during the postpartum estrus may range from 23 to 40 days depending, in general, upon the number of suckling young.[1–4] The prolongation of gestation is due to delay in implantation[5–8] and altered endocrine factors are responsible for this delay.[2–4,9] If certain hormonal conditions are satisfied implantation will occur, even in rats suckling large litters, at the same time (day 6) as in normal, non-lactating pregnant rats. The subsequent development of such embryos is normal and parturition occurs on the 23rd day.[2–4]

Methods. Each of 10 female rats was placed in a separate cage with a mature male the day before parturition was expected. Immediately after delivery each litter was adjusted to 9. In all animals spermatozoa were found in the vagina within 24 hours after parturition and the male was then removed. A pan on the bottom of the cage was filled to a depth of ½ inch with shavings used as nesting material. In each experiment .1 mg diethylstilbestrol in oil was shaken up in 20 cc of ether and then scattered about the cage. In all cases this was done on the day that spermatozoa were found and on such other days as indicated in Table I. The cages were not cleaned until the termination of the experiment.

Vaginal smears were taken routinely. Particular attention was directed to the appearance of the "placental sign." Free blood in the vagina gave evidence of resorption of some or all of the embryos. This was always confirmed by manual palpation of the uteri.

Controls consisted of 49 untreated rats each suckling 9 young. In these, the average day of implantation was 16.9 and the average length of gestation was 32.9 days.

Results. In one female (E228d) there was no indication of implantation or pregnancy. In the remaining 9 animals implantation occurred at a time (Table I) comparable to that of a normal pregnant rat. In 5 of these animals resorption began between days 9 and 12. In 2 animals (E233a and E230d) all but one of the embryos were resorbed between days 14 and 16. In each case the remaining embryo continued to develop but was resorbed between days 22 and 26. Two animals (E227f and E227b) delivered normal litters of 7 and 5 young respectively on day 23. The young were reared and were normal. Variations in total amount of hormone scattered about the cage did not have any obvious differential effects.

Discussion. Although the hormone was scattered about the cage, its entrance into the body of the mother is undoubtedly comparable to direct oral administration. Hormone adhering to the feet or fur of the mother

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7 Kirkham, W. B., *Anat. Rec.*, 1916, **10**, 219.
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TABLE I.
Effects of Scattering Diethylstilbestrol About the Cages of Inseminated Lactating Rats Suckling 9 Young.

Rat	Day																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
E226	x										R	R														
E227f	x		x																				B			
E227c	x		x		x				R	R	R															
E227e	x		x		x						R	R	R	R	R	R	R									
E228d	x		x		x̄																					
E230b	x		x		x					R	R	R	R	R	R											
E230d	x		x		x									R								R	R	R	R	R
E230a	x		x		x		x					R	R													
E233a	x		x		x		x		x					R	R	R						R	R			
E227b	x		x		x		x		x														B			

x—1 mg diethylstilboestrol scattered about cage.
R—Resorption of embryos taking place.
B—Normal litter born.

or to the skin of the pups would be licked off during the process of cleansing. Allen[10] has shown that natural estrogens are inactivated by the liver whereas stilbestrol is not. Thus the hormone, entering the circulatory system through the portal vein, should be as effective as if administered by injection. Results show that stilbestrol is capable of substituting for natural estrogen in reducing the time of implantation in inseminated lactating rats to normal. In certain cases the embryos may develop to term. The high percentage of resorptions is not surprising since administration of estrogen in exact quantities is not possible by this method and the antagonistic effect of large doses of estrogen on pregnancy is well known.

The study emphasizes the importance of isolating experimental animals that are being treated with stilbestrol. Other animals in the same cage may be profoundly affected and cause the investigator to draw erroneous conclusions from changes that might be observed. Cages should be cleaned thoroughly upon termination of experiments involving the use of stilbestrol.

Conclusions. Small amounts of diethylstilbestrol scattered about the cages of inseminated lactating rats suckling 9 young brings about implantation at the normal time (day 6) rather than around the 16th day as in untreated controls. The study emphasizes the importance of isolating experimental animals that are being treated with diethylstilbestrol.

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14251 P

Experimental Air-Borne Influenza Infection. I. Influence of Humidity on Survival of Virus in Air.*

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The ease with which infections with influenza A virus can be produced in susceptible animals by allowing them to breathe virus-containing air suggests that this may be an important mode of dissemination of the disease in man.[1-3] Its importance would depend on whether the virus survived in the air sufficiently long for an infective level to be built up. Previous studies have shown that the air in small chambers into which virus had been sprayed was infective for mice up to one hour.[3] Further studies have been made concerning the physical factors which influence the survival of virus in larger spaces simulating room environments. The data reported here include the effect of relative humidity on the persistence of virus in the air.

The experiments were performed in a room of 800 cubic feet capacity (10 x 10 x 8 feet). The air of the room was gently and continuously agitated by means of a centrally placed, slowly rotating vertical fan. The temperature varied from 27 to 29°C. The low humidity experiments were carried out during the winter months. The higher relative humidities were obtained by vaporizing steam into the room before each experiment. Influenza A virus (PR-8 strain) was employed and prepared from infected mouse lungs. The amount of virus used in each experiment varied from 2.4 to 3.6 cc of a 10^{-1} dilution by weight of ground lungs in 10% horse serum and broth. It was sprayed into the room with an atomizer which produced fine uniform-sized droplets. Following the atomization of the virus, groups of 10 mice were placed in the room for 20-minute periods at increasing intervals of time. As a more efficient means for the detection of air-suspended influenza virus 20 minute air samples (representing 20 cubic feet of air) were taken simultaneously with the Moulton air sampler.[4] The virus-containing broth was then inoculated intranasally into groups of 10 mice.† All mice dying were autopsied and examined for lung lesions. Those surviving were killed on the 12th to 14th day.

It was found that room atmospheres of high humidity (80-90%) into which virus suspension had been sprayed were no longer infective after one hour, although the mice exposed to this air 10 to 30 minutes after spraying all died with extensive consolidation of the lungs. Atmospheres of 45% to 55% relative humidity remained infective for 6 hours and were lethal for mice up to one hour and 10 minutes. At low humidities (17 to 24%) similar amounts of virus continued to produce influenzal pneumonia in the exposed mice for at least 24 hours. During the first 6 hours after introduction of the virus into this dry atmosphere all the mice died. At 12 hours 40% died and the remainder showed extensive pulmonary involvement. Among the mice exposed at 24 hours there were no deaths but pulmonary lesions were present in 30% of them. Experiments illustrating the influence of relative humidity on the per-

* This investigation was aided in part through the Commission on Cross Infections in Hospitals, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U. S. Army.

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† The recovery of influenza virus from the air by this method will be described in a separate publication by M. Hamburger, Jr., and C. G. Loosli.

Persistence of Influenza Virus in Air at Different Relative Humidities.

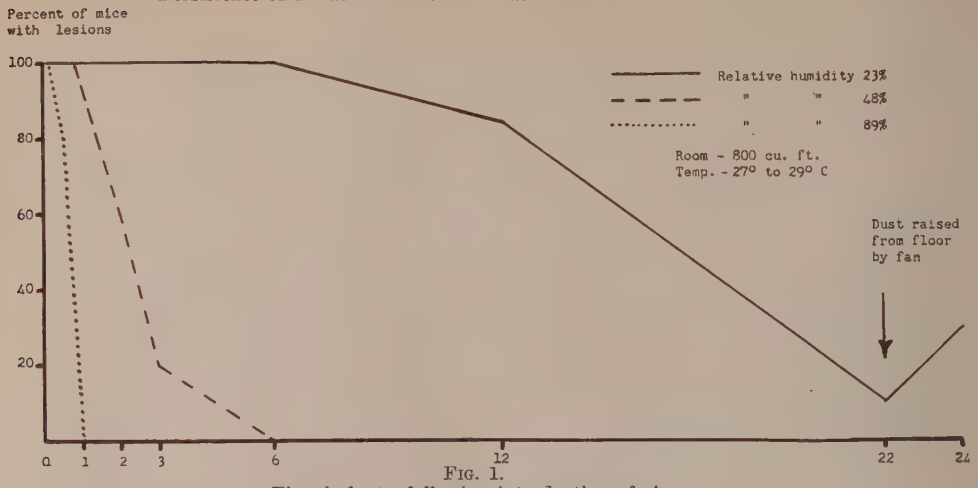

FIG. 1.

Time in hours following introduction of virus.

sistence of viable influenza virus in the air are shown graphically in Fig. 1.

In order to determine whether prolonged sojourn in atmospheres containing relatively low concentrations of influenza virus would result in a higher percentage of infections than occurred in mice exposed for the customary 20-minute period, groups of mice were placed in the experimental room 24 hours after introduction of virus into dry air and allowed to remain there for 2 hours. All these mice exhibited widespread consolidation of the lungs at autopsy while only one-third of the animals exposed for 20 minutes became infected and their lesions were limited in extent. These and other experiments reported in an earlier paper[3] indicate that intrapulmonary dosage which depends principally on the concentration of the infectious agent in the air and the amount of air inhaled, plays a very important role in determining the inception and severity of air-borne influenzal infection.

Our studies suggest that the disappearance of the virus from atmospheres of high humidity is due more to inactivation of the virus in the air rather than to a rapid settling out of the infectious particles as Edward and his colleagues[5] conclude, although both factors probably play a role. Evidence for the prolonged suspension of virus particles in the air was provided by the persistence of a marked Tyndall effect 12 hours after the introduction of the virus spray into an atmosphere of high humidity at a time when the air was no longer infective for mice. Further corroboration of the persistence of fine droplets in air of high relative humidity was derived from experiments in which mice exposed in the experimental room 4 hours following dispersal of streptococci, Group C, into the air, all became ill and died of streptococcal infection. Also large numbers of streptococci could be collected on settling plates and with the Moulton sampler for as long as 12 hours. The survival of the virus in atmospheres of low humidity is probably due to the rapid drying of the infectious particles as is indicated in the experiments of Edward.[6] Another indication of the survival of influenza virus in desiccated form was provided by certain experiments in which vigorous sweeping of the floor of the experimental room, 22 hours after spraying in the virus, enhanced the infectivity of the air. (Fig. 1). Preservation of viruses, for experimental purposes, by quick drying procedures gives further support to this view. These observations have an important bearing on the spread of infectious agents by the aerial route.

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14252

Influence of Previous Diet on Insulin Tolerance.*

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A number of workers have studied the relationship of diet to insulin tolerance.[1-6] The results obtained have been quite contradictory due to the fact the food intake was in no case equal and adequate for all groups of experimental animals. In the present experiments we applied to this problem the method of stomach tube feeding previously described by Reinecke, Ball and Samuels.[7] We have been able to demonstrate that shifting a large proportion of the dietary calories from carbohydrate to fat, keeping the total calories constant, results in an increased tolerance to insulin.

Methods. Adult male rats, obtained from Sprague-Dawley and weighing 250-300 g, were used. All animals were first force-fed a diet prepared by mixing equal amounts of the high fat and high carbohydrate diets described in Table I. Four days later, when the rats had become accustomed to feeding by stomach tube, they were separated into 2 groups having similar ranges of weight. One group was fed the high carbohydrate diet (83% of the calories as carbohydrate, 2% fat, 15% protein); the other group was given the high fat diet (85% of the calories as fat, 15% protein). The amounts fed were calorically equal in both groups, and were sufficient to maintain or increase body weight.

TABLE I.
High Fat and High Carbohydrate Diets Fed in Equi-Caloric Amounts by Stomach Tube.*

Constituent	High fat Amount	High Carbohydrate Amount
Lactalbumin (Labco No. 15-42)	15 g	15 g
Osborne-Mendel Salt Mix.	4 "	4 "
Celluflour (Chicago Dietetic)	10 "	10 "
Vitamin B Complex, Lederle	1 capsule	1 capsule
Percomorph Oil	1 drop	1 drop
Corn Oil	37.5 g	1 g
Dextrin, Merck's N.F.V.	0	83 "
Gum Ghatti	3.6 "	0
Water to make a total volume of 180 cc in each		

* Valuable aid in the development of these diets was furnished by Dr. R. M. Reinecke.

Adequate vitamin and mineral supplements were present in both diets.

After 6 weeks on this regime, the animals were fasted 36 hours and their sensitivity to exogenous insulin determined. The dosage of insulin (Lilly's Iletin) used was 0.16 U. per 100 g body weight injected intraperitoneally. Samples of blood, 0.05 ml in amount, were taken from the clipped end of the tail just before insulin injection and at intervals thereafter. Protein was precipitated by 5 ml of Folin's dilute tungstic acid reagent. Blood sugar determinations were made in duplicate on 2 ml aliquots of the tungstic acid filtrate by a modification of the Folin-Malmros micro-method. The procedure was similar to that described by Jeghers and Myers,[8] somewhat modified and adapted to the Evelyn photoelectric colorimeter.

Results. Fig. 1 illustrates the effect of previous diet on the insulin tolerance of the adult male rat. It is evident that after a 36-hour fast, blood sugar levels are similar

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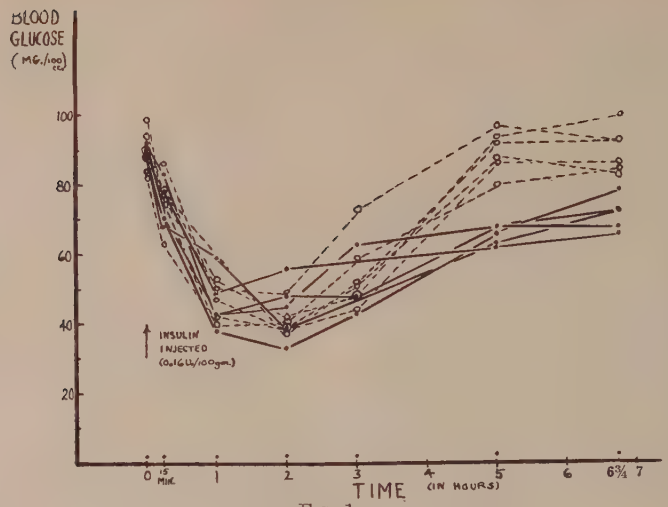

FIG. 1.

Diet and Insulin Tolerance of Normal Rats.

Blood sugar curves of normal adult male rats injected with 0.16 U. of insulin per 100 g after 36 hours fast. These animals had previously been fed by stomach tube for 6 weeks equi-caloric amounts of the special diets:

High carbohydrate diet •————•

High fat diet ○– – – – –○

Each curve represents one insulin test in one rat.

in both carbohydrate-fed and fat-fed animals. Likewise, the degree of depression of the blood sugar level by injected insulin is about the same. The recovery rates are, however, quite different. Animals previously force-fed the high fat diet show a consistently more rapid recovery from hypoglycemic levels.

Discussion. The above results show that the insulin tolerance of rats previously fed a high fat diet is greater than that of high carbohydrate-fed animals, after a 36-hour fasting period. Samuels, Reinecke and Ball[9] have shown that fat-feeding has a sparing action on liver glycogen disappearance during fasting. After 33 hours fasting, the liver of fat-fed animals contained much more glycogen than that of carbohydrate-fed animals. It seems likely then, that the difference in insulin sensitivity noted is a result of the difference in fasting levels of liver glycogen.

When insulin was injected, a rapid and equal depression of the blood sugar level occurred in both groups, indicating that the action of insulin in increasing carbohydrate utilization was unaffected by previous diet. In response to hypoglycemia, the liver liberated sugar by glycogenolysis. Since more glycogen was available in the fat-fed animals after the period of fasting used here, the rate of recovery from insulin hypoglycemia was more rapid.

Summary. Adult male rats, previously force-fed a high fat diet by stomach tube in amounts sufficient to maintain body weight, were less sensitive to the action of injected insulin than similarly-treated animals previously force-fed equi-caloric amounts of a high carbohydrate diet. The decreased sensitivity manifested itself primarily as a markedly increased rate of recovery from insulin hypoglycemia. The most likely direct cause of the increased insulin tolerance in the fat-fed animals is the higher level of liver glycogen after a 30-36 hour fasting period.

[9] Samuels, L. T., Reinecke, R. M., and Ball, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 456.

14253

Response of Hypophysectomized Immature Male Rats to Pregnant Mare Serum.*†

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Hypophysectomized mature and immature male rats will respond to pregnant mare serum as shown by the maintenance or restoration of spermatogenic function and the enlargement of the accessory reproductive organs.[1–5] In previous studies on hypophysectomized rats, the adult was generally used and treatment extended for a long period of time. The investigation here reported was undertaken to study the effects of pregnant mare serum on hypophysectomized immature male rats that were all subjected to a short (5-day) injection period. This study was of interest in view of results previously obtained in hypophysectomized immature female rats in which it was shown that dosage variation alone would produce markedly different types of ovarian stimulation.[6,7] Furthermore, Fevold[8] uses the male rat as an assay method for luteinizing hormone as determined by seminal vesicle weight increase.

Immature male rats ranging from 30 to 36 days of age were hypophysectomized. Body weights ranged from 71 to 86 g at the time of operation whereas the body weight at the time of autopsy (10 days later) ranged from 65 to 83 g. An interval of 5 days was allowed to elapse after hypophysectomy in all cases. The hormone was injected subcutaneously once daily for 5 days and the animals autopsied 24 hours after the last injection. All pituitary capsules were serially sectioned, stained with Mallory's connective tissue stain and examined microscopically. Only data from completely hypophysectomized animals are presented. Weights of the adrenals, testes and seminal vesicles (including the coagulating gland and any contained fluid) were taken.

Pregnant mare serum (Gonadin)‡ was administered in 6 different total doses to groups of 7 to 13 animals each. Testis weight increased in most animals receiving hormone when a comparison is made with untreated hypophysectomized rats, 10 days postoperatively. However, when a comparison is made with the testis weight of animals 5 days postoperatively, at which time injections were started, it is obvious that the effect of the hormone is essentially that of maintenance (Table I). Spermatids and spermatozoa were absent.

The lowest dose of hormone tested caused a 100% increase in seminal vesicle weight. Administration of larger doses resulted in a more pronounced response. However, it should be pointed out that the 25 and 50 r. u. total dosages were equally effective in their action on seminal vesicle weight (Table I).

It has been shown that PMS when administered to hypophysectomized immature female rats in a manner identical with the methods used here in males will produce a varied ovarian effect depending upon the

* Supported by a grant from the Penrose Fund of the American Philosophical Society.

† Grateful acknowledgment is made to Mr. Jacob P. Halperin for valuable technical assistance.

1 Evans, H. M. Meyer, K., and Simpson, M. E., *Mem. Univ. Calif.*, 1933, **11**, 257.

2 Smith, P. E., *J. Am. Med. Assn.*, 1935, **104**, 553.

3 Greep, R. O., *Cold Spring Harbor Symposium*, 1937, **5**, 136.

4 Liu, S. H., and Noble, R. L., *J. Endocrinology*, 1939, **1**, 7.

5 Pencharz, R. I., Cole, H. H., and Goss, H., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 432.

6 Leathem, J. H., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 590.

7 Cole, H. H., Pencharz, R. I., and Goss, H., *Endocrinology*, 1940, **27**, 548.

8 Fevold, H. L., *Cold Spring Harbor Symposium*, 1937, **5**, 93.

‡ Pregnant mare serum, Gonadin, was generously supplied by Dr. D. H. Wonder, Cutter Laboratories, Berkeley, California.

TABLE I.
Influence of Pregnant Mare Serum on Hypophysectomized Immature Male Rats.

Total dose	No. of rats	Avg organ wt (mg ± E_m) Testes	Sem. ves.
0	7	231	7.6 ± 0.6
2.5	10	443	14.9 ± 2.1
5.0	13	335	17.9 ± 2.9
12.5	7	273	19.3 ± 3.0
25.0	9	409	30.7 ± 2.8
50.0	11	366	29.9 ± 4.0
100.0	10	451	41.8 ± 4.5
Control rats 5 days after hypophysectomy		365	7.5
Unoperated control rats 35 days of age		718	13.2

E_m = mean deviation of the mean.

dosage injected.[6] A low dose (10 r. u.) causes marked thecal luteinization with little growth of follicles. A larger dose (25 r. u.) results in the development of large vesicle follicles and slight thecal luteinization whereas further dosage increases (50 r. u.) causes the formation of corpora lutea. The lot of hormone used in these experiments produced the same effect in the hypophysectomized female rats as those just described. It was of interest to find that a 25 r. u. dosage which produces a 10-fold ovarian weight increase and primarily a follicle stimulating effect also causes a 4-fold increase in seminal vesicle weight in hypophysectomized male rats. These results indicate that the potency of a dosage of pregnant mare serum as determined by its action on the ovary does not indicate its ability to increase seminal vesicle weight.

Summary. Pregnant mare serum maintains testis weight and stimulates seminal vesicle weight in hypophysectomized male rats treated for a 5-day period. These data are compared with those from similarly treated female rats.

14254

Reproducible Diuresis and Chloruresis for Bioassay of Antidiuretic Activity.

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For the bioassay of antidiuretic activity it is essential that control observations shall provide a diuresis which is both reproducible and quantitative. In a previous communication[1] certain results obtained by a satisfactory method were given. It is the purpose of this paper to describe in greater detail a comparison of the various procedures explored. In agreement with the prediction of Silvette[2] it appears that extracts of the posterior lobe of the pituitary gland can be assayed more accurately by measuring urinary chloride excretion than by measuring the inhibition of water excretion.

The rat method of assaying the antidiuretic activity of extracts of the posterior lobe of the pituitary gland was introduced by Burn.[3] Subsequently many modifications have been described both in the manner of producing hydration and in the calculation of results. These procedures have been employed to assay the antidiuretic activity of urine, blood serum and pituitary extracts.

Three different methods of hydration were chosen for comparison. (A) Gilman and Goodman[4] administered a preliminary "hy-

* This work done during tenure of a Commonwealth Fund Fellowship, 1940-41.

1 Ham, G. C., and Landis, E. M., *J. Clin. Invest.*, 1942, **21**, 455.

2 Silvette, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 599.

3 Burn, J. H., *Quart. J. Pharm. and Pharmacol.*, 1931, **4**, 517.

4 Gilman, A., and Goodman, L., *J. Physiol.*, 1937, **90**, 113.

drating" dose of water amounting to 2.5% of the rat's body weight by gavage 3 hours before beginning the assay. Then at the beginning of the assay period a second dose of water amounting to 5% of the body weight was administered. (B) Silvette[5] administered a total of 10 cc of 0.2% sodium chloride solution intraperitoneally per 100 g of rat. (C) Kreiger and Kilvington,[6] using rats weighing approximately 200 g, administered to each 12 cc of water by gavage.

The control diuresis produced by each of the 3 hydrating procedures was in turn measured and calculated by 4 different methods. (a) Burn[3] determined the average time required for the rat to excrete 50% of the total fluid administered. (b) Robinson and Farr[7] recorded the average time required for the rat to excrete a volume of urine equaling 2.5% of the initial body weight. (c) Silvette[5] determined the total volume of urine in cc excreted per 100 g of rat in the 6 hours elapsing after the fluid was administered. (d) Kreiger and Kilvington[6] plotted the amount of urine against time at 15-minute intervals for 5 hours and measured the area subtended by this curve. Antidiuretic agents prolonged the time required to reach the chosen endpoints in methods a and b, reduced the volume of urine excreted in 6 hours in method c, and reduced the area of the curve in method d.

In the comparative study here described new rats maintained under carefully controlled conditions of feeding, housing and hydration as described previously[1] were used in each experiment. The 3 methods of hydration (A, B, C, above) and 4 methods of analysis (a, b, c, d, above) were performed 3 times, using 2 groups of 6 rats in each experiment. The results of each group were averaged and then a grand average was computed for all 6 groups. Each figure therefore includes results on 36 rats or six groups of

TABLE I. Results of the 3 Methods of Hydration and the 4 Methods of Measurement and Calculation of the Diuresis Produced.

	a. 50% excretion time in minutes			b. Time in min. to excrete volume of urine equal to 2.5% of body wt			c. Total cc urine excreted in 6 hr			d. Area in square inches					
										6 hr			3 hr		
	Avg Min	Max. deviation %	Mean deviation %	Avg Min	Max. deviation %	Mean deviation %	Avg cc	Max. deviation %	Mean deviation %	Avg sq. in.	Max. deviation %	Mean deviation %	Avg sq. in.	Max. deviation %	Mean deviation %
A. Gilman and Goodman. Water equal to 2.5% and 5.0% of body wt by gavage.	73	29	9.6	64.6	27.8	11.3	5.73	7.8	4.7	24.5	4.9	3.3	18.61	10.7	5.5
B. Silvette. 10 cc 0.2% NaCl sol. intraper. per 100 g wt	196	22	14.3	120	36	10.8	7.01	22	9	24.2	26.8	10	11.43	34.9	11.6
C. Kreiger and Kilvington. 12 cc water by gavage.	124	21	12	82	19.5	10.4	8.8	17	6.6	32.7	18.3	6.9	18.64	23.7	12.8

[5] Silvette, H., *Am. J. Physiol.*, 1940, **128**, 747.

[6] Kreiger, V. I., and Kilvington, T. B., *M. J. Australia*, 1940, **1**, 575.

[7] Robinson, F. H., Jr., and Farr, L. E., *Ann. Int. Med.*, 1940, **14**, 42.

6 each. The mean deviation and maximum deviation from the final average were determined for all 6 groups and are shown in Table I.

These results showed that the method of double hydration described by Gilman and Goodman (A) and the area method of measurement of the resulting diuresis described by Kreiger and Kilvington (d) yielded the most reproducible results. In order to shorten the 6-hour period of observation required by this method so that 2 runs could be completed in one day the data were recalculated on the basis of the areas subtended by a curve plotted for 3 hours. The maximum and mean deviation for this shorter period are shown for each method of hydration in Table I. Despite the somewhat larger variation, the 2-dose technic was superior and for practical reasons was chosen for routine use in subsequent studies on the antidiuretic activity of human urine already reported.[1]

In preliminary observations, using known amounts of pituitrin,† it was found that the 3-hour area method described was quite accurate with doses of 0.5 to 10 milliunits of pituitrin per 100 g of rat. Doses larger than this produced curves with areas slightly greater than those for 10 milliunits and hence paradoxically smaller numerical values for antidiuresis. Examination of these curves revealed that doses exceeding 10 milliunits per 100 g of rat produced a brief diuresis and then secondarily, a profound antidiuresis, whereas the smaller doses produced antidiuresis only. This effect has been described before with larger doses and may be related to the temporary rise in blood pressure produced by pituitrin. Since the expression of results in terms of area includes the total effect, it is essential, with pituitrin at least, to dilute the sample of unknown potency to the proper range to obtain accurate quantitative results.

Although the larger doses of pituitrin had a paradoxically smaller antidiuretic activity over the three-hour period chosen their effect on the excretion of chloride in the urine, or their "chloruretic"‡ activity, increased steadily as the dose of pituitrin increased over a range of 0.1 to 100 milliunits per 100 g of rat. In fact comparison showed that pituitrin solutions may be assayed more accurately by measuring their effect on chloride excretion, than by measuring their antidiuretic activity alone. Table II shows both the antidiuretic effect and the chloruretic effect of solutions containing 0.1 to 100 milliunits of pituitrin per 100 g of rat when administered intraperitoneally in a total volume of 1 cc of physiological saline per 100 g of rat. Six groups of 3 rats were used in each assay; 2 groups as saline controls and 2 groups for each concentration of pituitrin. As previously described[1] the antidiuretic effect is expressed as the difference in square inches between the areas subtended by the curves of the test substance and the saline controls. The total chloride excretion of each group, per 100 g, during the 3-hour period of observation, was measured by the open Carius method of the Volhard titration as described by Van Slyke and Sendroy.[8]

TABLE II.
Antidiuretic and Chloruretic Effects of Pituitrin Solutions Ranging from 0.1 milliunits to 100 milliunits per 100 g of Rat.

Pituitrin in milliunits per 100 g of rat	Antidiuretic effect—difference in square inches from controls	Total chloride excreted—microequivalents per 100 g of rat per 3 hr
780 saline controls	± 0.78	Less than 90.0
0.1	+ 1.2	74.25
0.5	— 1.5	109.0
1.0	— 3.2	121.0
2.5	— 5.3	219.0
5.0	— 9.2	252.0
10.0	—10.3	273.0
25.0	— 4.9	362
50.0	— 5.3	436
75.0	— 5.0	512
100.0	— 4.7	653

Assay of pituitrin solutions containing from 0.5 to 10 milliunits per 100 g of rat is quite accurate using antidiuresis as the cri-

† Obstetrical pituitrin, Parke Davis, Lot No. 3260960, 1 cc containing 10 international units.

‡ Chloruretic: Gr. chloros, yellowish green; plus Gr. ouresis, urination; urination of chlorides.

8 Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry. Methods*, Williams & Wilkins, Baltimore, 1932, p. 835.

terion but with greater concentrations this method is grossly inadequate. On the other hand, the chloruretic effect increased regularly over the entire range of concentrations of pituitrin studied. In 130 experiments using 2 control groups of 3 rats each or a total of 780 rats the chloride excretion in the urine never exceeded 90 microequivalents per 100 g of rat per 3 hours. Thus definite sensitivity is shown with 0.5 milliunits per 100 g of rat.

Summary. (1) Three different methods of hydrating rats were explored and the diuresis produced was measured in 4 separate ways.

(2) The most reproducible results were obtained by hydrating with 2 doses of water and by recording diuresis as the area of the excretion curve.

(3) Pituitrin solutions, over a considerable range of concentrations, could be assayed more accurately by measuring their chloruretic effect than by measuring their antidiuretic effect.

14255 P

Excretion of Androgens, 17 Ketosteroids and Estrogens in the Dog Following Administration of Androgens.*

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Injection of testosterone propionate in large therapeutic doses is followed in humans by excretion of considerable amounts of androgenic material in the urine.[1-3] Such treatment is also followed by increase of estrogenic potency of the urine.[1,2,4,5] In contradistinction to the human, no estrogen and only traces of androgen were found in the urine of the dog after the injection of androgens.[6,7]

Since we are conducting an extensive study of the metabolism of estrogens and androgens in the dog it was felt necessary to reëxamine the urinary excretion of androgens and estrogens following the administration of androgens in this species.

Methods. Urine samples were collected in metabolism cages in 24-hour periods. In many instances four 24-hour samples were collected and pooled. Androgens and estrogens were extracted following hydrolysis with HCl at pH 1. Androgens were assayed on one-day-old cockerels by a modification of the method of Frank *et al.*[8] The urine extracts were applied directly to the comb in ether solution as suggested by Koch.[9] Estrogens were assayed on mice by vaginal smear and histological methods after Fluhman.[10] 17 Ketosteroids were determined by the method of Holtorff and Koch.[11]

Hormone excretion of the normal dog. Excretion of androgens, estrogens and 17 ketosteroids was studied in 3 female and 8 male dogs. Androgen excretion ranged from 150 to 350 γ per 24 hours, expressed as androsterone. One female dog excreted 1500 γ per 24 hours. 17 ketosteroid excretion ranged from 500 to 1500 γ per 24 hours,

* Aided by a grant from the Johnson Research Foundation, New Brunswick, N.J.

† J. E. Mears Fellow in Physiology and Medicine.

1 Dorfman, R. J., and Hamilton, J. B., *J. Clin. Invest.*, 1939, **18**, 67.

2 Hoskins, W. H., Coffman, J. R., Koch, F. C., and Kenyon, A. T., *Endocrinol.*, 1939, **24**, 702.

3 McCullagh, E. P., *J. Am. Med. Assn.*, 1939, **112**, 1037.

4 Steinach, E., and Kun, H., *Lancet*, 1937, **2**, 845.

5 Hamilton, J. B., and Dorfman, R. J., *J. Lab. Clin. Med.*, 1942, **27**, 917.

6 Kochakian, C. D., *Endocrinol.*, 1937, **21**, 60.

7 Kochakian, C. D., *Endocrinol.*, 1938, **23**, 463.

8 Frank, R. T., Klemperer, E., Hollander, F., and Kriss, B., *Endocrinol.*, 1942, **31**, 63.

9 Koch, F. C., *Assn. for Study of Internal Secretions*, 25th meeting, Atlantic City, 1941.

10 Fluhman, C. F., *Endocrinol.*, 1934, **18**, 705.

11 Holtorff, A. F., and Koch, F. C., *J. Biol. Chem.*, 1940, **135**, 377.

expressed as androsterone. There was no difference in androgen or 17 ketosteroid excretion between male and female dogs. No estrogenic activity was detectable in the urine of dogs of either sex.

Excretion of androgen and estrogen following injection of testosterone propionate. Two male dogs received daily injections of 10 mg testosterone propionate‡ in sesame oil for 12 days. Urine was collected (a) prior to injection, (b) during the last 4 days of treatment, and (c) one week after treatment had been discontinued. There was no increase in androgenic activity and no increase in 17 ketosteroid values in the urine. One of the two dogs excreted 10 i.u. per 24 hours of estrogenic material during the last 4 days of treatment. One week later no estrogenic activity was demonstrable. In experiments to be reported in another communication, large single doses of androgenic compounds (testosterone, androsterone, methyltestosterone) were given intravenously in alcoholic solution. Estrogens were found in the urine in 5 of 7 experiments (40, 40, 24, 6 and 100 i.u. per 24 hours respectively). In one experiment, intraduodenal administration of testosterone was followed by estrogen excretion in the urine. No increased excretion of androgens was observed in any of these experiments.

Androgen and estrogen excretion following the injection of testosterone propionate into castrate male dogs. The 2 male dogs in which sex hormone excretion following treatment with testosterone propionate had been studied were castrated. Several weeks later the dogs received another course of 12 injections of 10 mg testosterone propionate. No androgen was excreted in the urine. The dog which had excreted estrogen during treatment with testosterone propionate prior to castration did so again after castration.

Androgen and estrogen excretion following injection of testosterone propionate into the adrenalectomized female dog. A female dog was adrenalectomized by the usual 2-stage operation and was maintained with desoxycorticosterone acetate. Androgen excretion prior to adrenalectomy was 150 γ per 24 hours. Following adrenalectomy no androgens were detectable. The dog was then given 12 daily injections of 10 mg testosterone propionate. During the last 4 days of treatment with testosterone, traces of androgens (8 γ per 24 hours) were excreted. At this time 10 i.u. of estrogens were excreted per 24 hours, while there had been no estrogen excretion either before or after adrenalectomy.

Discussion and Summary. In contradistinction to the human, urine androgens do not increase in the dog following administration of androgenic hormones. These findings confirm earlier observations by Kockakian.[6] Administration of androgens also fails to increase the excretion of 17 ketosteroids. However, contrary to earlier findings by Kochakian,[7] dogs were found to excrete estrogens following administration of androgens, as has been found in humans.[1,2,4,5] This excreted estrogen may be either formed from the administered androgen or may be the result of stimulation of some source of estrogen production. The fact that estrogens are excreted in the castrated and in the adrenalectomized dog following testosterone treatment narrows considerably the possible sources of the estrogens. Conversion from the administered androgen seems more likely but is not yet definitely proven.

‡ Testosterone, testosterone propionate, methyltestosterone and desoxycorticosterone acetate were generously supplied by Dr. E. Schwenk and Dr. M. Gilbert of the Schering Corp., androsterone by Mr. R. Mautner of the Ciba Corp.

14256

Effect of Certain Drugs on Properties of the Human Atrioventricular Node.

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In reciprocal rhythm an impulse originating in the atrioventricular junctional tissue passes to the ventricle, and in addition by retrograde conduction stimulates the atrium. If the retrograde conduction has been rapid, the cycle terminates; however, if conduction has been slowed, the impulse may find the junctional tissue responsive, and after traversing part of the auricle again stimulate the A-V node and ventricle, producing a reciprocal beat, or "Umkehr-Extrasystole." Clinical examples of such a mechanism were first described by White,[1] who based his concept of the physiological processes present on the circus mechanism observed earlier by Mines.[2] Reciprocal rhythm has been reported by others, and has been discussed most recently by Scherf.[3]

We have recently studied a patient who manifested this type of disturbance, and have taken the opportunity to study the effect of several drugs on the properties of the junctional tissue, presumably in the neighborhood of the A-V node. The clinical features of the case will be described elsewhere, together with certain electrocardiographic findings that may be of theoretical importance. The possibility suggested by Luten and Jensen[4] that some of the reported cases represent a parasystole rather than a reciprocal rhythm, has in this instance been definitely excluded.

By measuring the RP intervals of a large number of complexes, we have determined the retrograde conduction time below which reciprocal stimulation of the ventricle did not occur, but above which reciprocal beats were seen. This value we have taken to represent the refractory period of the junctional tissue. The mean value of the RP interval has been taken as a measure of retrograde conduction; the corresponding PR interval for a given RP time (0.40 sec.) has been taken as a measure of forward conduction. Curves relating RP and PR intervals have shown recovery of the conduction tissue;[5] this recovery time has been regarded also as the time of relative refractoriness.[6]

The effects of drugs, and of nervous influences, on the physiological properties of cardiac tissues have been studied extensively since the early studies of Lewis and his associates; the most modern studies are those of Macleod[7] and of Wedd *et al.*[8] It seems superfluous to review them here.

Method. Electrocardiograms were taken first by means of an esophageal electrode, since the clear-cut P waves obtained in this fashion facilitated the measurement of the RP and PR intervals. Since passage of the esophageal electrode sometimes appeared to influence reflexly the rate and the site of the pacemaker, a CF lead was used routinely, with the chest electrode placed in the third interspace at the right sternal border; this lead gave satisfactorily sharp P waves. The records were taken for a period of 5 to 8 minutes. After a control curve, a drug was administered; at the estimated time of full effect, indicated in Table II, further tracings were recorded. Measurements were made with a hand lens.

Results. In Table I we show the changes with varying amounts of *digitalis* studied over a period of 25 days. The quantity of digitalis accumulated has been estimated in

1 White, P. D., *Arch. Int. Med.*, 1915, **16**, 517.

2 Mines, G. R., *J. Physiol.*, 1913, **46**, 370.

3 Scherf, D., *Arch. Int. Med.*, 1941, **67**, 372.

4 Luten, D., and Jensen, J., *Am. Ht. J.*, 1931-32, **7**, 593.

5 Lewis, T., and Master, A. M., *Heart*, 1925, **12**, 209.

6 Ashman, R., *Am. J. Physiol.*, 1925, **74**, 121.

7 Macleod, A. G., *Am. Ht. J.*, 1939, **17**, 294, 305.

8 Wedd, A. M., Blair, H. A., and Gosselin, R. E., *J. Pharm. and Exp. Therap.*, 1942, **75**, 251.

TABLE I.
Digitalis Action on A-V Node.

Date	Digitalis cumulation, cat units	Rate, per min	Refractory period, sec	Forward conduction, sec	Retrograde conduction, sec	Recovery
Feb. 16	0	50	0.26	0.185	0.30	Complete in 0.45 sec.
25	3	48	.285	.195	.18	Complete in 0.45 sec.
Mar. 4	5	45	.37	.255	.18	Delayed and incomplete.
5	5	48	.375	.27	.25	More delayed.
6	5	50	.36	.255	.40	
9	5	50	.36	.27	.41	Further delayed and least complete.
11	7	48	.40	.285	.38	Less delay and more complete.
12	6	52	.32	.25	.22	Very slight delay; almost complete.
13	5	51	.29	.205	.15	Normal again.

TABLE II.
Drug Action on A-V Node.

Date		Time after drug	Rate per min	Refractory period, sec	Forward conduction	Retrograde conduction	Recovery
Mar. 5	Control		48	0.375	0.27	0.25	
	Prostigmin	10 min.	45	.47	.34	.36	Delayed and incomplete
	Atropine	1 "	53	.32	.245	.21	Return to control curve
6	Control		50	.36	.255	.35	
	Atropine	1 "	50	.36	.275	.27	No definite change
11	Control		48	.40	.285	.38	
	Epinephrine	8 "	50	.39	.28	.44	Slightly more complete
	Neosynephrine	17 "	47	.39	.285	.27	No change
12	Control		52	.32	.25	.22	
	Quinidine	1 hour	46	.46	—	.49	Marked delay; never complete
		2 "	44	.41	.315	.35	Less delay; finally complete
13	Control		51	.29	.205	.15	
	Mecholyl and atropine	2 min	33	*.48-.76	—	.50	Marked delay; not quite complete
		7 "	50	.36	.24	.30	Less delay; not quite complete
		20 "	52	.31	.23	.23	Return almost to control curve

* All retrograde conduction times of 0.48 sec or less showed no reciprocal beats; all above 0.76 showed reciprocal beat; none between these figures.

the usual fashion, and must be regarded as approximate only. The refractory period of the junctional tissue, and forward conduction time through this area, vary in a manner which closely parallels the estimated digitalis saturation. Retrograde conduction shows first an acceleration with smaller doses, and then a delay with larger doses of digitalis. This is similar to the findings of Clark and Mines[9] in their study of the effects of digitalis on the frog heart.

[9] Clark, A. J., and Mines, G. R., *J. Physiol.*, 1913-14, **47**, vii.

Recovery curves after digitalization show delay, *i.e.* longer PR intervals for given RP intervals, and failure to become complete, *i.e.*, longer PR intervals even with the longest RP periods. These changes seem to follow closely the prolongation of the refractory period, which suggests that changes in relative refractoriness parallel the effective refractory period. This appeared to be true of digitalis effect, as well as of the effects produced by the other drugs employed.

We could detect no phase of supernormal conduction.

In Table II are summarized the effects of other drugs. Prostigmine (1 mg., i.m.) prolonged the refractory period and slowed both retrograde and forward conduction. This effect was more than neutralized by the subsequent injection of atropine (1/50 grain i.v.), though atropine alone on the following day showed a less conspicuous effect.

Epinephrine (0.5 cc, subcu.) showed a definite effect only by slowing retrograde conduction, while neosynephrin (5 mg. subcu.) did little more than accelerate it. Both produced very slight shortening of the refractory period.

Quinidine (5 grains, p.o.) markedly prolonged the refractory period and slowed A-V conduction in both directions. One hour after administration, forward conduction could not be tabulated since the refractory period exceeded the time (0.40 sec.) selected as the constant RP interval at which the PR time was measured.

An uncomplicated mecholyl (acetyl-B-methyl choline 20 mg subcu.) effect was not obtained since the reaction following its injection necessitated the immediate administration of atropine. From the curves taken, however, there may be made out easily the marked prolongation of refractoriness and delay in conduction, which gradually regress under the influence of atropine.

Further studies were prevented by the spontaneous disappearance of the reciprocal mechanism, which we were unable to reproduce.

Comment. We consider that the indicated changes in the refractory period are the result of medication, and that the rate change contributes no important part of this effect. The rate change was trivial in most instances; at the slow rates observed the refractory period changes relatively little with change in rate; and finally, we could measure the refractory period of the ventricle—that is, the QT interval with varying Q-Q periods—and found virtually no change due to slight variations at the slower rates.

The effective refractory period of the junctional tissue is definitely prolonged by digitalization; this is in agreement with Lewis' earlier work,[10] but must be interpreted in the light of the later studies of Drury,[11] Macleod,[8] and Wedd,[12] in which they conclude that the local absolute refractory period of heart muscle is shortened by digitalis glucosides. The delay of A-V conduction is commonly recognized clinically, though the initial acceleration of retrograde conduction finds it parallel only in animal experiments.

The depressant action on heart muscle of quinidine is illustrated by its effect on conduction, on recovery, and on refractoriness. Prolongation of the latter is in agreement with views long held, but which have not been confirmed in the latest study of Wedd *et al.*;[12] however, in the intact heart of our subject we cannot clearly divorce refractoriness from conduction effects, and are measuring the effective, rather than the absolute local refractoriness.

Carotid sinus stimulation was ineffective in our patient, but mecholyl and prostigmine produced the striking effects noted above. We note the total effect and do not feel justified in attempting the separation of direct effects from those mediated through the vagus, if indeed such a distinction is valid.

It is noteworthy that the susceptibility to change, and the magnitude of change, is in every instance much greater for retrograde conduction than for forward conduction.

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[10] Lewis, T., Drury, A. N., and Iliescu, C. C., *Heart*, 1921-22, **9**, 21.

[11] Drury, A. N., and Love, W. S., *Heart*, 1926, **13**, 77.

[12] Wedd, A. M., Blair, H. A., and Dwyer, G. K., *J. Pharm. and Exp. Therap.*, 1941, **72**, 394.

14257

Significance of Variations of Prothrombin Activity of Dilute Plasma.

C. E. Brambel and F. F. Loker. (Introduced by W. R. Amberson.)
With the technical assistance of E. J. Curtis.
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The introduction of the hemorrhagic principle, Dicoumarol, (3,3′ methylenebis (4-hydroxycoumarin)) has supplied a new impetus to the study of the prothrombin mechanism of plasma. Several investigators[1-4] have suggested that dilute plasma may be a more sensitive indicator of variations in prothrombin activity and consequently may be more efficaciously used to follow Dicoumarol therapy. During the course of this investigation on prothrombin activity of serial dilutions of human plasma, data have been obtained which suggest a physiological basis for clinical conditions manifesting an intravascular disturbance of the coagulation mechanism. Quick's one stage method applied to dilute plasma showed a difference in coagulation activity of "normal" plasma and plasma obtained from individuals having intravascular disturbances.

Prothrombin activity curves for "normal" plasma and plasma obtained from individuals in various clinical conditions were constructed by serial dilution with physiological saline. Quick's[5] method was followed meticulously in every detail. The clotting time for oxalated undiluted plasma (12-15 seconds) was repeatedly corroborated with regard to the narrow limits of variation that occur and found to be in complete agreement with the above mentioned investigator. However, with 12.5% and 25% plasma the clotting time deviated markedly from the values reported by Quick, indicating less prothrombin activity for these dilutions. Reproducible activity curves have been obtained from 60 "normal" individuals by the present method. Fig. 1 shows the limits of variation for "normal" human plasma for all dilutions compared with Quick's published data. It is to be noted that the greatest difference was encountered when 12.5% plasma was used. The factors responsible for the difference found are being subjected to a critical study.

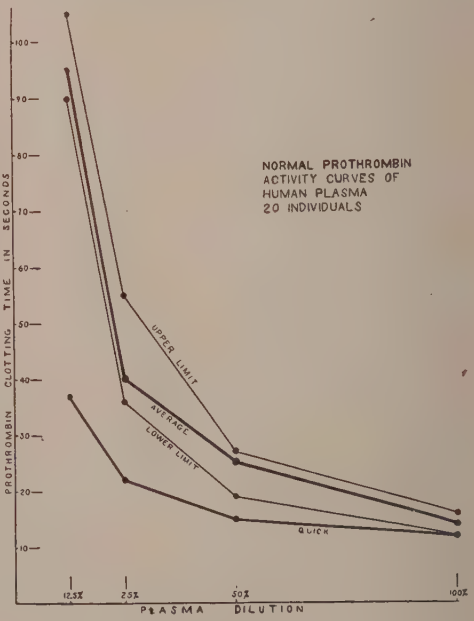

Fig. 1.

Comparison of Quick activity curve with average "normal" curve obtained in the present study. Abscissæ represent plasma dilutions with physiological saline and ordinates prothrombin clotting time in seconds. The curve showing the greatest activity was obtained from Quick's data, and deviates markedly from the values obtained in the present investigation. The limits of variation are indicated by thin lines.

1 Stahman, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

2 Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

3 Wright, I. S., and Prandoni, A., *J. Am. Med. Assn.*, 1942, **120**, 1015.

4 Shapiro, S., and Sherwin, B., *N. Y. State J. Med.*, 1943, **43**, 45.

5 Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Charles C. Thomas, Springfield, Illinois, 1942.

TABLE I.

	Prothrombin clotting time, sec			
	Undil. plasma	12.5% plasma	No. individ.	
Normal	12-14	90±10	60	
Phlebitic	12-14	60±10	30	
Maternal	12-14	50±10	50	*
Fetal	15-18	110±20	50	*
Preoperative	12-14	90±10	5	†
3-5 days postoperative	12-14	60±10	5	†
10-14 " "	12-14	90±10	5	†

* Maternal and fetal blood taken simultaneously at time of delivery.
† Same individuals.

Increased plasma prothrombin activity expressed in terms of decreased clotting time was observed in certain clinical conditions when 12.5% plasma was used while undiluted plasma gave the established normal value. The data presented in Table I shows that an acceleration of the clotting power of dilute plasma (12.5%) was found in phlebitis, post-operative surgical trauma and during childbirth.

Individuals during childbirth reveal the most striking results. The undiluted maternal plasma and plasma obtained from the umbilical cord at the time of delivery have nearly identical prothrombin clotting time values; 12.5% maternal plasma under the same conditions coagulate with a markedly accelerated rate (50-60 seconds) while cord (fetal) blood plasma has a normal clotting time (110 ± 20 seconds). On the basis of the preceding data, it is suggested that a protective physiological mechanism may be set up by the maternal organism producing plasma prothrombin activators leading to a decreased clotting time during this critical period. This protective mechanism may function as part of a hormonal system antecedent to hemorrhage at the time of delivery. Hormones as factors affecting the coagulation system may be an explanation for the phenomenon observed. The fetal circulation, on the other hand, is not interrupted, consequently excess prothrombin activators are not essential and a normal prothrombin activity curve results. Fig. 2 shows the limits of variation for maternal and fetal plasma from 50 deliveries. A marked difference in activity curves between the two plasmas can be observed and furthermore, the limits of variation do not overlap except for undiluted plasma.

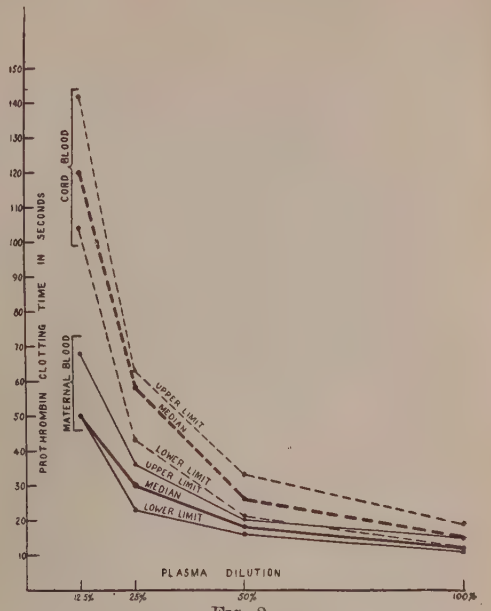

FIG. 2.

These curves show the limits of variation for maternal and fetal plasma from 50 deliveries. A marked difference in activity curves between the two plasmas can be observed, and that the limits of variation do not overlap except for undiluted plasma. Maternal plasma shows an increased activity while fetal plasma exhibits essentially normal activity. Abscissæ represent plasma dilutions with physiological saline and ordinates prothrombin clotting time in seconds.

During post-operative surgical trauma a decreased clotting time for dilute plasma (12.5%) was found for all individuals studied. After 10 days the accelerated coagulation was restored to the normal condition. (Table I). During this period, prothrombin activators may be produced in excess as part of a protective mechanism of the organism in response to hemorrhage.[6] Failure of the accelerated mechanism to return to normal may undoubtedly be one of the contributory causes leading to clinical post-operative thrombophlebitis. The phenomenon following post-operative surgical trauma is similar

[6] Cannon, W. B., *The Wisdom of the Body*, W. W. Norton, New York, 1939.

to that observed in childbirth.

Individuals with clinical symptoms of phlebitis manifest a decreased clotting time for dilute plasma (12.5%) and a normal value for undiluted plasma (Table I). In this clinical condition the prothrombin activators may possess a potency sufficient to produce intravascular clots and embolus formation. Silberberg[7] postulates biochemical factors that may be responsible for accelerated coagulation. It is of special interest in this connection that patients receiving the hemorrhagic principle, Dicoumarol, exhibit intravascular coagulation difficulties when the effects of the drug cease and the prothrombin clotting time of 12.5% plasma again shows a decreased coagulation time compared with a normal individual.

Dicoumarol* has been used to disrupt the prothrombin mechanism intravascularly. Individuals with chronic clinical phlebitis do not respond as readily as normal individuals to this hemorrhagic agent and return in a short time to their previous condition. Dicoumarol therapy can be followed most satisfactorily when dilute plasma (12.5%) is used. Variations in clotting time can be detected with this dilution that are not manifest when undiluted plasma is used. No toxic symptoms were observed in any of the individuals receiving this hemorrhagic principle.

Since the foregoing observations demonstrate increased prothrombin activity of the plasma in some pathological states, an attempt was made to induce this condition in "normal" individuals by intravenous injections of repeated large doses of a synthetic vitamin K derivative (Hykinone).† No increase in coagulation activity was found in any of the dilutions of normal plasma. These findings are in keeping with all previous observations that vitamin K derivatives do not accelerate the prothrombin coagulation mechanism above normal limits but rather activates an organ, *i.e.*, the liver, to produce this thrombin precursor until an optimum amount is available for normal processes.

Quick's simple one stage procedure for evaluating the prothrombin clotting time of oxalated plasma has been successfully adapted to provide an index to the prothrombin activity occurring in the various above mentioned clinical conditions. By this means, employing dilute plasma (12.5%), it is possible to measure accelerated coagulability of plasma. The demonstration of prothrombin "activators"[7] as a protective mechanism or as a pathological state is suggested to account for the difference in clotting activity encountered. The nature of these "activators" is being subjected to a critical biochemical and physiological study.

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7 Silberberg, M., *Physiol. Rev.*, 1938, **18**, 197.

* The Dicoumarol (3,3′-methylenebis (4-hydroxycoumarin)) used in this study was supplied by the Abbott Laboratories, Chicago, Ill.

† The vitamin K derivative (Hykinone) was supplied through the generosity of the Abbott Laboratories, Chicago, Ill.

14258

Lowest Barometric Pressure Compatible with Life in an Atmosphere of 100 Per Cent Oxygen.

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Armstrong[1] has stated that life is impossible at an altitude where the barometric pressure equals 87 mm Hg even in an atmosphere of 100% oxygen. He explains this conclusion by assuming the water vapor tension in the lungs to be 47 mm Hg and the carbon dioxide tension to be 40 mm Hg. Hence, at an altitude where the barometric pressure corresponds to 87 mm Hg no oxygen would be able to enter the alveoli of the lungs even though one were breathing pure oxygen. However, it is generally known that hypernea occurring at high altitude considerably reduces the carbon dioxide tension in the alveoli. Moreover, the water vapor tension, too, tends to fall during hyperventilation.[2] These observations suggest that the theoretical minimal barometric pressure compatible with life in an atmosphere of 100% oxygen is lower than given by Armstrong. The present investigation aims to show that this is true.

Ten male rats weighing approximately 250 g were subjected to decreased barometric pressure in an atmosphere of 100% oxygen. The barometric pressure was reduced by a vacuum pump which permitted a constant flow of oxygen. In the first 5 minutes the pressure was gradually lowered to 180 mm Hg, in the next 5 minutes to 90 mm Hg and thereafter by 5 mm Hg every 4 minutes until gasping occurred. Soda lime was placed in the chamber to absorb carbon dioxide. The rate of reducing the pressure was controlled by an outside manometer connected to the decompression chamber, while the lower values of pressure were read from a closed manometer placed directly in the chamber. The experiment was terminated when gasping occurred; restoration to normal atmospheric pressure revived the animals in several minutes.

TABLE I.
Barometric Pressure at Which Respiratory Failure Occurred in Rats in an Atmosphere of 100% Oxygen.

Rat	Group A	Group B (allowed to hyperventilate for an additional 20 min)
1	60	50
2	65	70
3	65	55
4	65	60
5	75	50
6	65	65
7	70	65
8	65	65
9	60	60
10	60	

In a second group (9 animals) the same procedure as above was carried out except that the animals were kept for 20 minutes at a pressure varying between 75 and 90 mm Hg, depending on the pressure at which the greatest degree of hyperpnea occurred. After this period the decompression was carried on to the gasping state as in the first experimental group. This was done to allow additional time for reducing the carbon dioxide and water vapor tensions.

As shown in the accompanying table, the average barometric pressure at which gasping occurred in an atmosphere of pure oxygen was 65 mm Hg with variations between 60 and 75 mm Hg. In the second group permitted to hyperventilate for an additional 20 minutes the average pressure for gasping was 60 mm Hg with variations between 50 and 70 mm.

Observations of Bert[3] and Selladurai and Wright[4] suggest that at a partial pressure of about 10 mm Hg of oxygen in the alveoli

1 Armstrong, H. G., *Principles and Practice of Aviation Medicine*, Baltimore, 1939, Williams and Wilkins Co.

2 Christie, R. V., and Loomis, A. L., *J. Physiol.*, 1932, **77**, 35.

3 Bert, P., *La Presson Barometrique*, 1878.

4 Selladurai, S., and Wright, S., *Quart. J. Exp. Physiol.*, 1932, **22**, 233.

death occurs. If this value of 10 mm Hg for oxygen is subtracted from 50 mm Hg, the lowest barometric pressure at which gasping was found to occur in an atmosphere of pure oxygen, 40 mm Hg remain for the partial pressures of carbon dioxide and water vapor. It is evident that these pressures must fall considerably below the values given by Armstrong. The fall in the partial pressure of carbon dioxide is due, as is well known, to the hyperventilation occurring during anoxia and the fall in water vapor tension is probably the result of two factors: (a) lowering of body temperature as a result of anoxia[5] and (b) the lack of equilibrium between alveolar air and blood as a result of the increased ventilation.[2]

Summary. Two groups of male rats were subjected to a progressive reduction in barometric pressure while in an atmosphere of pure oxygen in order to discover the pressure at which respiratory failure occurred. The first group gave an average value of 65 mm Hg with variations between 60 and 75 mm Hg, and the second group, allowed to hyperventilate for an additional 20 minutes, gave an average value of 60 mm Hg with variations between 50 and 70 mm Hg. The difference between the two groups shows that the additional hyperventilation had a slight effect on reducing the atmospheric pressure at which respiratory failure occurred in this experiment. These results indicate that the partial pressures of carbon dioxide and water vapor fall lower than previously assumed and therefore life is possible at a higher altitude in the presence of pure oxygen than heretofore believed.

5 Gellhorn, E., *Am. J. Physiol.*, 1937, **120**, 190.

14259

Effects of Yeast Extracts and Phenylmercuric Nitrate on Yeast Respiration and Growth.

ELTON S. COOK AND CORNELIUS W. KREKE. (Introduced by S. Tashiro.)

From the Research Laboratories of the Institutum Divi Thomae, Cincinnati, Ohio.

In earlier papers it has been pointed out that yeast extracts having the property of increasing respiration are able to offset the toxicity of phenylmercuric nitrate and of *n*-butyl-*p*-hydroxybenzoate for molds[1] and to overcome the depressant effects of phenylmercuric nitrate on skin respiration without loss of inhibitory power for *S. aureus* in the concentrations employed.[2] We had also found that, with certain fractions from yeast, ability to stimulate the oxygen uptake of yeast paralleled ability to increase yeast proliferation.[3]

In continuation of our studies on respiration depression as an index of toxicity and on the relation between respiration and growth we have investigated the effects of phenylmercuric nitrate on the growth and respiration of yeast and the ability of yeast extracts to modify those effects.

Respiration experiments were carried out at 37.5°C by the previously described Warburg manometric technic,[4,5] using a suspension containing 1.56 mg (dry weight) of Fleischmann's bakers' yeast in Ringer-phosphate-glucose (0.02% glucose, pH 7.2). Aqueous-alcoholic yeast extracts were pre-

1 Cook, E. S., and Kreke, C. W., *Nature*, 1940, **146**, 688.

2 Cook, E. S., Kreke, C. W., Eilert, M. R., and Sawyer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 210.

3 Cook, E. S., Hart, M. J., and Stimson, M. M., *Biochem. J.*, 1940, **34**, 1580.

4 Cook, E. S., Kreke, C. W., and Nutini, L. G., *Studies Inst. Divi Thomae*, 1938, **2**, 23.

5 Cook, E. S., and Morgan, M. N., *Biochem. J.*, 1940, **34**, 15; Cook, E. S., Walter, E. M., and Eilert, M. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 547.

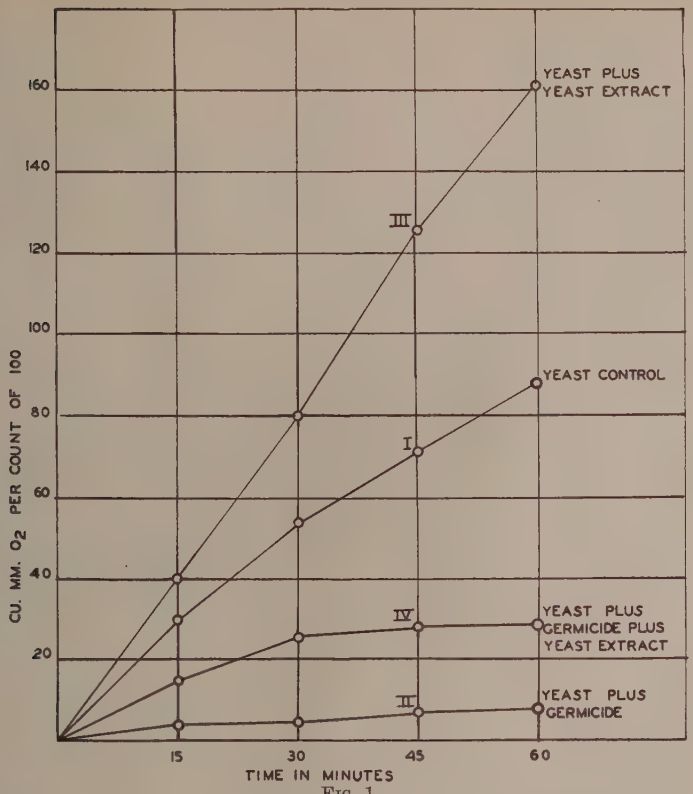

FIG. 1.

Effects of Phenylmercuric Nitrate and Yeast Extract on Yeast Respiration.

pared as described previously[4] from Fleischmann's bakers' yeast and corresponded to crude Fraction A of the earlier work.[4]

Fig. 1 shows the result of a typical experiment on the effects of phenylmercuric nitrate and yeast extract on the respiration of bakers' yeast. Each point represents the average of duplicate determinations which agreed within 5% or better. Curve I represents the normal respiration of the yeast suspension. In Curve II, phenylmercuric nitrate sufficient to give a concentration of 1:100,000 was tipped in from the side arm immediately after the equilibrium period. Very little oxygen uptake was observed and the cells were completely killed at the end of the hour experimental period as determined by methylene blue staining. In Curve III, yeast extract to give a final concentration of 1 mg per cc was tipped in and gave an immediate increase in oxygen consumption amounting to 86% at the end of the hour. All cells were living at this time. Curve IV shows the result of adding a mixture of phenylmercuric nitrate and yeast extract to give the same final concentrations. The respiration, while considerably depressed, continued for 45 minutes, after which further oxygen uptake ceased. Protection was afforded by the extract.

In other sets of experiments, the phenylmercuric nitrate was added at the beginning of the experiment and the extract was tipped in after 30 minutes; or the extract was added at the beginning of the experiment and the germicide after 30 minutes. In the former case, the extract exerted no protective action detectable either by respiratory or methylene blue criteria. In the latter case, the respiration, which was initially increased above the control, fell below it upon addition of the

germicide and steadily decreased in rate until respiration had almost ceased after 45 minutes, although no cells were dead by the methylene blue method. Again, protection is indicated. As a further check on the viability of the cells at the end of the experiment, a loop-full was taken from each respirometer flask and was plated out on sterile Sabouraud's medium and incubated at 30°C over night. Suspensions to which the germicide had been added after prior exposure to the extract gave as good growth as the controls, whereas the suspensions which were first exposed to the phenylmercuric nitrate gave no growth.

During the preparation of the yeast extracts, certain fractions were obtained which were without effect on the oxygen uptake of yeast or which had only an extremely slight effect (20% stimulation after 1 hour). These extracts were ineffective in offsetting the toxic action of the phenylmercuric nitrate as shown by respiration, methylene blue and plating criteria.

The effects of the yeast extract in counteracting the inhibitory action of phenylmercuric nitrate on the growth of yeast was demonstrated as follows. Petri plates were prepared containing solid Sabouraud's medium with different concentrations of phenylmercuric nitrate. In half of the plates the medium also contained 1% of yeast extract. Each plate was inoculated in the center with a loopful of a 72-hour culture of a pure strain of *Saccharomyces cerevisiae* isolated from Fleischmann's bakers' yeast. After 3 days of incubation at room temperature, the area of the yeast growth was measured with a planimeter. Results of a typical experiment are given in Table I.

TABLE I.
Effect of Phenylmercuric Nitrate and Yeast Extract on Growth of *S. cerevisiæ*.

Dilution of phenylmercuric nitrate	Colony area in in²	
	No extract	1% extract
1:10,000	.001	.003
1:20,000	.001	.003
1:30,000	.005	
1:40,000	.004	.008
1:50,000	.004	.008
1:100,000		.020
1:200,000	.008	.033
No phenylmercuric nitrate	.020	.047

Discussion. The results reported demonstrate that an aqueous-alcoholic yeast extract will protect bakers' yeast from the toxic action of phenylmercuric nitrate. This protection may be evaluated by respiration measurements, thus confirming earlier findings with regard to respiration as an index of toxicity.[1,2,6] However, after the respiration rate has fallen to zero, the yeast cells, under the conditions of the experiments, are still viable, as indicated by the methylene blue and plating experiments. Greig and Hoogerheide[6] have pointed out that bacteriostasis may occur without alteration of metabolic rate. We have found that yeast extracts not only increase oxygen uptake but also increase fermentation of yeast suspensions, and that "wound hormone" preparations from yeast, while very active in increasing yeast proliferation, have relatively little effect on yeast respiration.[7] It is well known that certain growth factors for yeast, such an pantothenic acid[8] and biotin,[9] markedly influence the metabolism of yeasts deficient in these substances. On the other hand, we have found the respiration of unstarved bakers' yeast and of the pure strain of *S. cerevisiae* derived therefrom to be relatively little affected by most of the bios components.[5,7,10]

[6] Adams, P. D., *Arch. Dermatol. and Syphilol.*, 1936, **36**, 606; Cook, E. S., *Chem. Products*, 1939, **1**, 65, and 1939, **2**, 89; Amersbach, J. C., Nutini, L. G., and Cook, E. S., *Arch. Dermatol. and Syphilol.*, 1941, **43**, 948; Manifold, M. D., *Proc. Roy. Soc. Med.*, 1940, **33**, 12; Bronfenbrenner, J., Hershey, A. D., and Doubly, J., *J. Bact.*, 1939, **37**, 583; Ely, J. D., *ibid.*, 1939, **38**, 391; Greig, M. E., and Hoogerheide, J. C., *ibid.*, 1941, **41**, 549, 557; Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 249.

[7] Cook, E. S., and Cronin, A. G., *Studies Inst. Divi Thomae*, 1941, **3**, 205.

[8] Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, 1939, **22**, 637.

[9] Burk, D., Winzler, R. J., and du Vigneaud, V., *J. Biol. Chem.*, 1941, **140**, xxi.

[10] Cook, E. S., Walter, E. M., Rack, F. J., Eilert, M. R., and Sawyer, M. A., *Studies Inst. Divi Thomae*, 1941, **3**, 147.

Summary. Phenylmercuric nitrate in a dilution of 1:100,000 is toxic to suspensions of bakers' yeast as shown by respiration measurements, methylene blue staining, and plating. One per cent of an aqueous-alcoholic yeast extract protects yeast to some extent from this germicidal action. Respiration may cease after introduction of yeast extract and phenylmercuric nitrate, but the yeast is still viable as shown by methylene blue staining and plating tests. The importance of respiratory depression in the mechanism of phenylmercuric nitrate toxicity is supported, but caution must be exercised in the use of respiratory depression as a sole criterion of toxicity for yeast. The yeast extract overcomes the growth-depressant effects of phenylmercuric nitrate on yeast.

14260

Comparison of Effects of Cobra Venom and Morphine on Unanesthetized Cat.

DAVID I. MACHT.

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The effect of morphine on cats and other felines differs radically from that which it exerts on man and other higher animals in two respects. (1) Instead of depressing the brain and neuromuscular activity, it produces excitation and delirium. (2) Instead of causing myosis, morphine dilates the pupil. Since cobra venom is employed clinically as an analgesic[1-4] it was desirable to compare its effects with those of morphine on unanesthetized cats because all previous studies on cats had been made under general anesthesia. In the present paper a comparison was made of (1) behavior and neuromuscular activity and (2) the pupils of unanesthetized cats after administration of the respective drugs.

Twenty cats were employed in this research. Inasmuch as the same animals were used repeatedly after complete recovery from successive injections, over 70 experiments were performed. The cobra venom employed was that made in these laboratories, free from hemotoxic and cytotoxic principles as well as proteins and virtually a solution of cobra neurotoxin. This solution, assayed on mice, was injected intramuscularly in doses ranging from 5 to 20 m. u. per kilo of cat. Morphine was used in the form of sulfate.

Behavior. While morphine induces in cats an excitement which increases with size of the dose and lasts for 18 hours or more, cobra venom has an exactly opposite action. Doses of 5 to 10 m. u. per kilo of cobra venom had little or no effect on cats. Occasionally they effected a mild euphoria causing the cats to roll pleasurably on their backs in the same way in which they react to catnip. Larger doses of cobra venom (10 to 20 m. u.) were sedative. The animals lay quietly and drowsed but roused to a touch. Very large doses (40 m. u. per kilo) of cobra venom were more depressant and caused unsteadiness of gait and tremor. The behavior of cats injected with cobra venom is apparently the reverse of their reaction to morphine, which induced excitement corresponding to size of the dose. The mild primary stimulation supervening an hour after injection of cobra venom was followed by a sedation proportional to size of the dose and lasting over 18 hours.

Eyes. In all experiments on cat's pupils constant illumination was maintained and there was no abrupt exposure to direct light.

1 Macht, D. I., *Med. Rec.*, 1941, **153**, 369.

2 Chopra, R. N., and Chowhan, J. S., *Ind. Med. Gaz.*, 1940, **75**, 69.

3 Bullrich, R. A., and Ferrari, J. A., *Prensa Medica Argentina*, 1940, **27**, 12.

4 Barbeau, A., and Laurendeau, E., *J. de l'Hotel-Dieu de Montreal*, 1940, **9**, 114.

Experiment on Maltese Cat Weighing 2.8 Kilo., 5-14-43

12:00 m., width of pupils about 5mm; right, ● left, ●
12:10 p.m., injected 30 m.u. of cobra venom
12:40 p.m., definite sedation; cat quiet; pupils same width
2:00 p.m., pupils much narrower, 2 mm. wide; right, ● left, ●
3:00 p.m., cat somnolent, lying comfortably; pupils very narrow, slit-like, 1 to 2 mm. wide; right, ● left, ●
Next morning (5-15-43) behavior normal and both pupils, 3 mm., ●

Experiment on Black and White Cat Weighing 2.3 Kilo. 5-14-43

12:00 m., pupils 2 to 3 mm. wide; right, ● left, ●
12:15 p.m., injected 30 mg. of morphine sulphate in muscle
12:40 p.m., cat very restless and excited; pupils round and maximally dilated; right, ● left, ●
2:00 p.m., very excited, pupils maximally dilated, do not respond to light
3:00 p.m., cat restless, excited; pupils still maximally dilated.
Next morning (5-15-43) cat quiet but nervous. Pupils beginning to get smaller; 6 mm. each; right, ● left, ●

Cats injected with morphine invariably revealed mydriasis, even after such small doses as 1 mg per kilo though injection of that amount may not excite. Larger doses of morphine were followed by maximal dilatation of pupils, which did not contract in bright light. The effect of cobra venom was much different. Doses of less than 5 m. u. per kilo did not affect the size of the pupil while larger doses (5 to 20 m. u. per kilo) produced a definite narrowing of the transverse diameter of the cat's eyes, beginning usually an hour after injection of the venom and persisting often till next day. Doses of 20 m. u. per kilo induced marked myosis and slit-like pupils with a transverse diameter of one mm. The respective effects of cobra venom and morphine on cats' behavior and eyes are illustrated by the following protocols and drawings, which do not indicate absolute measurements but only relative sizes of their pupils.

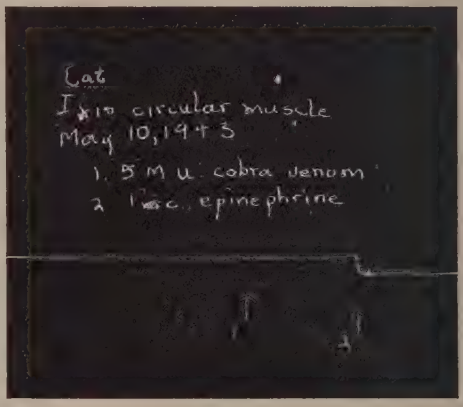

FIG. 1.
Effect of cobra venom and epinephrine on iris muscle.

Sollmann[5] gives in detail the usual explanation for myosis in *man* after morphine and ascribes it to a stronger constrictor oculomotor tone. The action is central. The

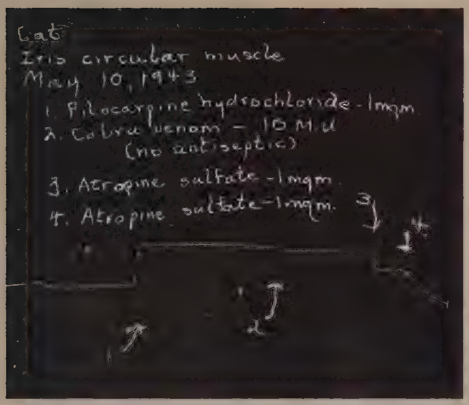

FIG. 2.
Effect of cobra venom and other drugs on iris muscle.

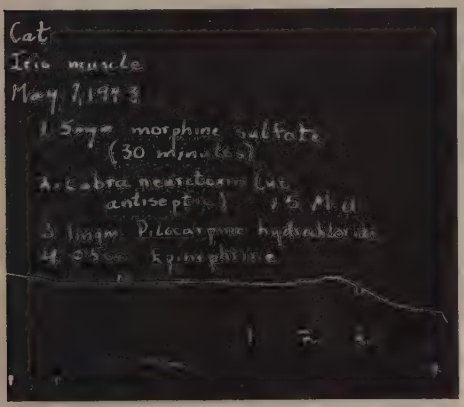

FIG. 3.
Effect of morphine and cobra venom on iris muscle.

oculomotor constrictor center is in a state of partial inhibition through a variety of sensory impulses. When this inhibition is released by such narcotics as morphine, the increase of oculomotor tone produces a myosis.

Local applications of cobra venom to the intact cats' eyes had no effect on the iris. To study the action of cobra venom on the pupil in greater detail the present writer experimented with the isolated iris muscle of cats *in vitro* by the method first described by Macht[6,7] and later employed by Poos[8] and then by Yonkman.[9] It was found that neither morphine (5 mg in 100 cc of Locke solution) nor cobra venom (10 m. u. in 100 cc of Locke solution) effected any change in tonus of the circular muscle. Larger doses, especially of *crude* cobra venom (1 mg in 100 cc of Locke), slowly relaxed the muscle by its cytotoxic action, yet the muscle still responded to such agents as ergotoxin. The data obtained reveal that the myosis cobra venom effects is not peripheral but central in origin, a finding corroborated by the results of tests which the author made on decerebrated cats with the assistance of Dr. M. B. Macht, neurophysiologist. In these experiments the upper brain was severed just below the thalamus while the oculomotor nuclei were left intact. Postoperative injection of cobra venom in such animals also *narrowed* the pupils.

Summary. 1. In unanesthetized cats morphine induces progressive excitation of the central nervous system and neuromuscular apparatus while cobra venom effects a sedation proportionate to dose of neurotoxin given. 2. While even small doses of morphine produce extreme dilatation of the pupil, cobra venom in small doses has little or no effect on its size though in larger doses it causes a definite myosis. 3. The evidence presented proves that this narrowing of cat's iris is due to a central action of the drug, which heightens the tonus of the oculomotor nucleus.

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7 Macht, D. I., Offener Brief, etc., *Arch. f. exp. Path. u. Pharm.*, 1938, **132**, 382.

8 Poos, F., *Arch. f. exp. Path. u. Pharm.*, 1927, **126**, 307.

9 Yonkman, F., *J. Pharm. and Exp. Therap.*, 1931, **40**, 195.

14261 P

Quantitation of Changes of Vasomotor Tone.* Change of Vasomotor Tone as Cause of Traube Hering Waves.

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This paper presents a technic for expressing quantitatively changes in vasomotor tone which has been applied successfully in a number of experiments. The description will, however, be limited to a single typical experiment in which the vasomotor tone was oscillating spontaneously (Traube Hering waves).

Method. The hind limb of an anesthetized dog was isolated at mid-thigh by two tight wire ligatures which compressed all structures except the femoral artery and vein and the sciatic and saphenous nerves. The dog was heparinized† and blood flow from a constant pressure reservoir into the femoral artery measured with a photographically recording orifice meter‡ at each of a series of perfusion pressures from 10 to 105 mm Hg. The femoral arterial (perfusion), femoral venous and aortic pressures were recorded simultaneously.

Results. The aortic pressure oscillated rhythmically between 100/80 and 120/100 at the rate of one cycle per 17 seconds; the venous pressure was 10 mm Hg. At perfusion pressures of 10 and 30 mm Hg, no flow occurred; at a pressure of 48 mm Hg the flow oscillated rhythmically from 0 to 0.7 cc/min., each increase and decrease of flow preceding slightly the associated decline and rise of aortic pressure. At higher perfusion pressures the flow was continuous but oscillated between maximum and minimum rates. The simultaneous measurements of flow (abscissal scale—blood flow cc/min.) and the difference of pressure between the femoral artery (perfusion pressure) and vein (ordinate scale AP-VP mm Hg) at moments of maximum (a, b, c, etc.) and of minimum (a^1, b^1, c^1, etc.) flow at each perfusion pressure are plotted in Fig. 1.

Discussion. Since the curves $P,F_{unconstricted}$, and $P,F_{constricted}$ drawn through the points of maximum and minimum flow respectively yield smooth divergent lines it is felt that the vessels assumed the same degree of constriction and dilation at the minimum and maximum points throughout the series of flow measurements. Therefore such pairs of curves express most satisfactorily the change of vasomotor tone. However, it is desirable in many experiments to find some numerical expression for the change of tone which can be plotted with respect to time or which can be used in comparing the results of one experiment with those obtained in another.

Alterations of vasomotor tone are often thought to parallel changes of peripheral resistance—defined as the ratio of pressure to flow. However, as may be seen from the curve $P,F_{unconstricted}$, the peripheral resistance changed from 27 mm Hg/cc/min. at a difference of pressure of 54 mm Hg to 8.6 mm Hg/cc/min. at a difference of pressure of 86 mm Hg in the absence of any active change of vasomotor tone. Furthermore, the peripheral resistances at point *c* in the unconstricted, and at point *g'* in the constricted state are approximately the same—50 and 54 mm Hg/cc/min. respectively—although the vasomotor tone is obviously much greater at *g'*.

Change of vasomotor tone has often been computed in terms of the ratio of flow in an unconstricted state to the flow in the constricted state when the vascular net is perfused at a constant head of pressure. In order for this procedure to have quantitative significance, this ratio should be the same regardless of the perfusion pressure used. However, as shown in Fig. 1 by the plot of

* Supported by a grant from the Commonwealth Fund.

† Liquaemin (Courtesy Roche Organon, Inc.)—0.3 ml/kg initially plus 0.1 ml/kg/half hour.

‡ Gregg, D. E., and Green, H. D., *Am. J. Physiol.*, 1940, **130**, 114.

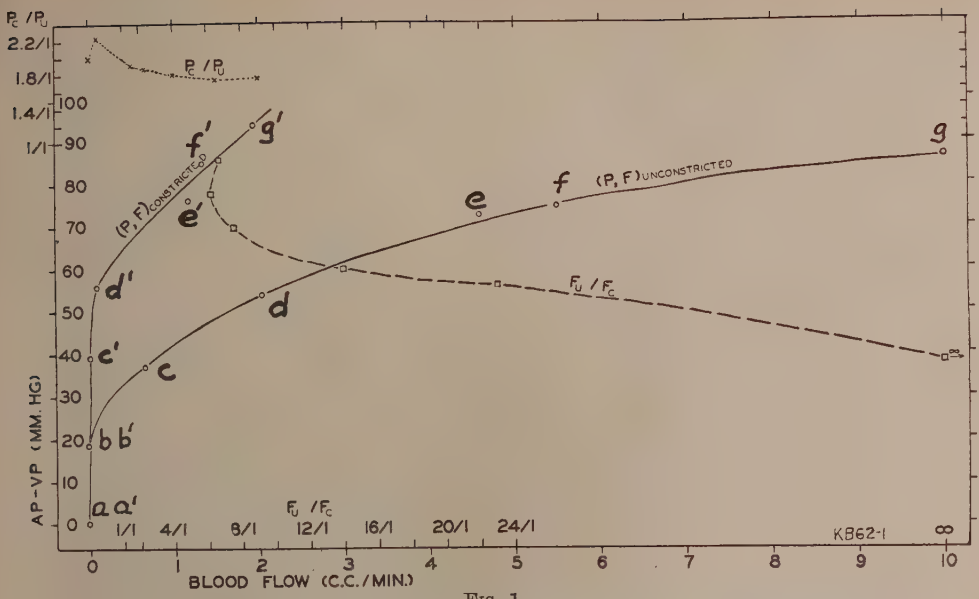

FIG. 1.

Plots of simultaneous measurements of the flow through the isolated vascular net of the femoral artery (blood flow cc/min) and of the difference of pressure between the femoral artery and vein (AP-VP.—mm Hg) of an anesthetized dog during the moments of maximum vasoconstriction $P,F_{constricted}$ and maximum vasodilation $P,F_{unconstricted}$, occurring during a continuous series of Traube Hering waves. a, a′; b, b′; c, c′; etc., are the corresponding maximum and minimum flows at each perfusion pressure.

Pc/Pu = plot of the ratio of the difference of pressure (AP-VP.—mm Hg) in the constricted state to the difference of pressure in the unconstricted state necessary to cause a given rate of flow (upper ordinate scale—Pc/Pu) vs. the rate of flow (abscissal scale—blood flow in cc/min).

Fu/Fc = plot of the ratio of the rate of flow in the unconstricted state to the rate of flow in the constricted state at each of several differences of pressure (abscissal scale — Fu/Fc) vs. the difference of pressure (ordinate scale — AP-Vp.-mm Hg).

Fu/Fc (abscissal scale) vs. the arteriovenous difference of pressure (ordinate scale), this ratio is approximately 6/1 at a difference of pressure of 86, but increases to 23/1 at a difference of pressure of 55 and to infinity at a difference of pressure of 40.

Comparison of the ratio of the difference of pressure in the constricted state to the difference of pressure in the unconstricted state necessary to cause the same flow indicates that this ratio varies only from 1.7/1 to 2.3/1 (see plot of Pc/Pu (ordinate scale) vs. flow (abscissal scale) in Fig. 1.) In view of the greater constancy of this ratio which has been confirmed in many other experiments, it is our belief that changes of vasomotor tone may best be expressed in terms of the ratio of the A-V difference of pressure in the constricted (or experimental) state to the A-V difference of pressure necessary to cause the same flow in the unconstricted (or control) state.

Computed by this method, it appears that the Traube Hering waves seen in this experiment were due to rhythmic changes in vasomotor tone which had a value of approximately 1.7/1 in the hind leg and recurred at the rate of one cycle/17 seconds.

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Antibacterial Effects of Quinones.*

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Fosdick, Fancher and Calandra[1] reported that small amounts of synthetic vitamin K (2-methyl-1,4-naphthoquinone) prevented the production of acid when saliva-glucose mixtures were incubated. Armstrong and Knutson[2] demonstrated that this effect of 2-methyl-1,4-naphthoquinone is due to the quinone structure of the compound and found that the same result could be obtained, in varying degree, with other naphtho-, tolu- and benzoquinones. Increased interest in the bacteriostatic and bactericidal influence of quinones is derived from the reports relating the activity of at least two antibiotic substances to quinones. Fumigatin (3-hydroxy,-4-methoxy,2:5-tolu-quinone) produced by *Aspergillus fumigatis,* possesses a definite ability to inhibit the multiplication of several gram-positive organisms.[3] Certain synthetic dimethyoxy quinones related structurally to fumigatin have been found to exert a remarkable antibacterial effect *in vitro*.[4,5] Waksman and Woodruff[6] note that actinomycin contains a quinone group and report a comparison of the bacteriostatic effect of tolu-p-quinone with that of several antibiotic agents. The same quinone was found[7] to exhibit a high degree of effectiveness in interfering with the luminescence of *Photobacterium fischeri* and a considerable bacteriostatic action against *Streptococcus pyogenes.*

The previous report[2] from this laboratory noted that several of the quinones which are effective in the prevention of acid formation in incubated saliva-glucose mixtures had been found to inhibit the growth of certain strains of streptococcus, staphylococcus and pneumococcus in liquid media. The present report presents quantitative data with respect to the required bacteriostatic and lethal concentrations of several quinones against two species of gram-positive pathogenic cocci. The coagulase-positive strain of staphylococcus was originally isolated from a patient with an osteomyelitic sinus. This strain was transferred daily in broth and was carried through at least 3 successive transfers before being employed in these experiments. After 14 transfers the culture was discarded and a fresh series of transfers were started from a colony on an agar plate. The streptococcus was strain C703 kindly supplied by Dr. Eleanor Bliss of the Johns Hopkins University Hospital. This strain was transferred twice daily in glucose broth and several times during the progress of the work it was reisolated from blood agar plates.

* This work was aided by a grant from the William John Gies Fund of the American College of Dentists and by a grant from the Carnegie Corporation. We also wish to express our thanks for gifts of material used in this work as follows: Dr. Louis F. Feiser, Harvard University (2-hydroxy1,4-naphthoquinone and 1,2-naphthoquinone-4-potassium sulfonate); Dr. Richard Kohn-Richards, Abbott Laboratories (Hykinone and 2-methyl-1,4-naphthoquinone); Dr. Oliver Kamm, Parke Davis (Synkamin); Dr. Walter M. Lauer, University of Minnesota (2,6-dimethoxy benzoquinone); Dr. Karl Folkers, Merck and Company (sodium 2-methyl-1,4-naphthohydroquinone disulfate); Dr. J. M. Carlisle, Merck and Company (penicillin); and Dr. Eleanor Bliss, Johns Hopkins University Hospital (the culture of C203 strain streptococcus).

1 Fosdick, L. S., Fancher, O. E., and Calandra, J. C., *Science,* 1942, **96**, 45.

2 Armstrong, W. D., and Knutson, J. W., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 307.

3 Oxford, A. E., and Raistrick, H., *Chemistry and Industry,* 1942, **61**, 128.

4 Oxford, A. E., *Chemistry and Industry,* 1942, **61**, 189.

5 Oxford, A. E., *J. Chem. Soc.,* 1942, p. 577.

6 Waksman, S. A., and Woodruff, H. B., *J. Bact.,* 1942, **44**, 373.

7 Rake, G., Jones, H., and McKee, C. M., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 136.

The quinones or other substances to be tested, which were conveniently soluble in alcohol, were made up in this solvent so that equal volumes contained equal molecular amounts of the several substances. The materials not soluble in alcohol were dissolved in sterile distilled water or an appropriate solvent† to produce solutions of the same molecular concentration as the alcoholic solutions referred to above. The required volumes (0.025 to 1.0 cc) of these solutions were transferred to sterile 25 cc volumetric flasks so as to produce in the final volume to be incubated definite molecular quantities of each substance. In the case of the experiments with the staphylococcus these quantities corresponded, on a molecular basis, to 0.125 to 5.0 mg of 2-methyl-1,4-naphthoquinone per 100 cc of the final dilution. The increment of concentration, expressed in the same manner, between flasks of each series was 0.125 mg between the first 2 flasks and 0.25 mg between subsequent flasks. The non-aqueous solvents were evaporated under reduced pressure in a vacuum desiccator causing the quinone to be deposited as a film over the wall of the flask. Sufficient water was added to all flasks so that the total volume of added water was 1.0 cc. The inoculum was prepared by adding 1.0 cc of a 10^5 dilution of a 24-hour broth culture of the staphylococcus to each 8.0 cc of a large volume of broth.‡ Nine cubic centimeters of the resulting dilution of organisms in broth were transferred to each of the 25 cc volumetric flasks, the same lot of bacterial suspension always being used to inoculate each of the flasks in a series set up to determine the bacteriostatic and lethal concentrations of a particular substance. The mouth of each flask was covered by a small sterile vial and the space between the neck of each flask and the vial was filled with a ring of cotton. The flasks were incubated with constant shaking at 37° for 4-5 hours and the incubation continued without agitation for a total of 48 hours. The solutions were examined for growth of the organisms after 24 and 48 hours of incubation by noting whether a turbidity distinct from the appearance of uninoculated broth was present. All solutions which appeared to exhibit no growth after 48 hours were tested for sterility by observing the growth, after 24 hours, on nutrient agar pour plates prepared with 1.0 cc of the solution in question. In critical cases sterility was also tested by inoculating several loopfuls of the suspected solution into 10 cc of broth.

In Table I is shown the minimum amounts of several quinones and other substances required to inhibit the growth of the staphylococcus for 24 hours and for 48 hours, and the quantities of the same substances required to kill the organisms present in the original inoculum. In several instances the results of duplicate experiments carried out on different dates are shown. In the case of a few substances, noted by the sign indicating "greater than", quantities equal to 29×10^{-6} mols per 100 cc (equivalent to 5.0 mg per 100 cc of 2-methyl-1,4-naphthoquinone) were totally ineffective against this strain of staphylococcus under the conditions of these experiments. Concentrations greater than 29×10^{-6} mols per 100 cc were not employed in this study since this amount is 10 times the quantity required to produce bacteriostatic and bactericidal results in the case of the most effective quinones.

Oxford[4] has previously reported 2,6-dimethoxy benzoquinone to inhibit the growth of a certain strain of staphylococcus in a dilution of 1:400,000 and was inferior in this regard only to 4,6-dimethoxy toluquinone. It is to be noted that 2-methyl-1,4-naphthoquinone equals, and the other naphthoquinones approach, the ability of 2,6-dimethoxy-benzoquinone to inhibit the growth or to kill the

† Water was the solvent for phenol, penicillin, and 1,2-naphthoquinone-4-potassium sulfonate; absolute alcohol for sulfathiazole; chloroform for 2,5-dichlorobenzoquinone and 2,6-dimethoxybenzoquinone; ether and alcohol for tetrachlorobenzoquinone. 2-Methyl-1-naphthol-4-amino hydrochloride and an equal quantity of sodium bisulfite were dissolved in water.

‡ The broth contained 10 g peptone (Cudahy's), 5 g extract of beef (Limco), and 5 g NaCl per 1000 cc. The final pH of various batches varied between 7.09 and 7.35. The 0.5% glucose broth had the same composition except for the addition of separately sterilized glucose and a final pH range of various batches of 7.3 to 7.6.

TABLE I.

In vitro Antibacterial Effects Against a Strain of Staphylococcus of Quinones and Other Substances Compared on a Molecular and on a Weight Basis.

		Minimum concentrations required for complete					
		Inhibition of growth for				Sterility after	
		24 hr		48 hr		48 hr	
Compound	No. Organisms	Mols per 100 cc x10⁶	Mg per 100 cc	Mols per 100 cc x10⁶	Mg per 100 cc	Mols per 100 cc x10⁶	Mg per 100 cc
2-Methyl-1,4-naphthoquinone	3,200	2.9	0.50	2.9	0.50	2.90	0.50
	2,030	2.9	0.50	4.35	0.75	4.35	0.75
2,6-Dimethoxy-benzoquinone	3,580	2.9	0.49	2.9	0.49	2.90	0.49
	2,030	2.9	0.49	4.35	0.73	4.35	0.73
1,4-Naphthoquinone	4,000	7.25	1.15	8.7	1.38	8.70	1.38
1,2-Naphthoquinone	4,200	4.35	0.69	8.7	1.38	10.15	1.60
2-Methyl-1,4-naphthohydroquinone	3,600	2.9	0.50	2.9	0.50	2.90	0.50
2-Methyl-1-naphthol-4-amino hydrochloride	3,500	2.9	0.61	4.35	0.91	4.35	0.91
Benzoquinone	3,600	>29.0	>3.14	>29.0	>3.14		
	1,650	8.7	0.94	8.7	0.94		
	1,500	7.25	0.78	8.7	0.94	8.70	0.94
Hydroquinone	3,420	5.8	0.64	7.25	0.80	7.25	0.80
	1,520	5.8	0.64	7.25	0.80	7.25	0.80
Tolu-para-quinone	4,040	>29.0	>3.58	>29.0	>3.58		
	2,240	14.5	1.77	14.5	1.77		
	1,500	23.2	2.83	29.0	>3.58		
Tolu-hydroquinone	2,780	5.8	0.72	7.25	0.90	8.70	1.08
	1,750	7.25	0.90	8.7	1.08	8.70	1.08
2-Hydroxy-1,4-naphthoquinone	2,750	>29.0	>5.03	>29.0	>5.03		
1,2-Naphthoquinone-4-potassium sulfonate	1,940	>29.0	8.03	>29.0	>8.03		
2,5-Dichlorobenzoquinone	1,820	23.2	4.11	>29.0	>5.14		
Tetra-Chlorobenzoquinone	2,300	11.6	2.85	11.6	2.85	11.6	2.85
Sulfathiazole	2,900	>29.0	>7.42	>29.0	>7.42		
Phenol	1,720	>29.0	>2.73	>29.0	>2.73		

strain of staphylococcus used in these experiments. The results obtained with 2-methyl-1-naphthol-4-amino hydrochloride (Synkamin of Parke Davis) are to be explained by its ready oxidation to the corresponding 1,4-naphthoquinone, a fact which also accounts for its vitamin K activity. The marked effect produced on antibacterial ability by substitution in the quinoid ring is seen when one compares the results obtained with 1,2-naphthoquinone *versus* 1,2-naphthoquinone-4-potassium sulfonate, and those obtained with 2-methyl-1,4-naphthoquinone and 1,4-naphthoquinone *versus* 2-hydroxy-1,4-naphthoquinone. Benzoquinone and toluquinone had previously been recognized as possessing a certain degree of antibacterial ability and this study shows that the corresponding hydroquinones exhibit equal or greater effects of the same sort. In the same connection, it is to be noted that 2-methyl-1,4-naphtho-hydroquinone possesses an antibacterial potency against this strain of staphylococcus equal to that of the corresponding quinone. While a broth medium containing peptone is not the optimum medium for the demonstration of the effectiveness of sulfathiazole against staphylococci, the results with this substance and with phenol show them to be less than one-tenth as effective, under the conditions of these experiments, as the most potent of the quinones.

Table II contains the results obtained in 2 groups of experiments in which a single suspension of staphylococci in broth furnished all the inocula for the experiments in each group. All solutions in each group of experiments, one using plain broth and the other glucose broth, were incubated concurrently. It is thus possible to compare the relative bacteriostatic effectiveness of the substances named in Table II under identical experimental conditions. The data given permit the calculation that the penicillin was

TABLE II.
Comparisons of Antibacterial Effects Against a Strain of Staphylococcus Substances Under Uniform Conditions of Inoculum.

Compound	Minimum concentrations required for complete: Inhibition of growth in plain broth* 24 hr: Mols per 100 cc x10⁶	Mg per 100 cc	48 hr: Mols per 100 cc x10⁶	Mg per 100 cc	Inhibition of growth in glucose broth† 24 hr: Mols per 100 cc x10⁶	Mg per 100 cc	48 hr: Mols per 100 cc x10⁶	Mg per 100 cc
2-Methyl-1,4-naphthoquinone	2.9	0.50	2.9	0.50	4.35	0.75	4.35	0.75
2,6-Dimethoxybenzoquinone	2.9	0.49	2.9	0.49	5.70	0.98	7.28	1.22
1,4-Naphthoquinone	5.8	0.92	5.8	0.92	10.15	1.60	13.06	2.06
1,2-Naphthoquinone	4.35	0.69	5.8	0.92				
2-Methyl-1,4-naphthol-4-amino hydrochloride	2.9	0.61	2.9	0.61	4.35	0.91	5.80	1.21
Tolu-hydroquinone					7.25	0.90	17.4	2.16
Penicillin	3‡		4‡					

* Size of inoculum—1,920 organisms.
† Size of inoculum—6,410 organisms.
‡ Florey units per 100 cc; one unit of material used equivalent to 0.0091 mg.

approximately 13 to 18 times as effective, on a weight basis, as 2-methyl-1,4-naphthoquinone or 2,6-dimethoxy benzoquinone. However, the sample of penicillin was probably not a pure substance and its molecular weight is undoubtedly higher than either of the two quinones mentioned above. Therefore, the actual effectiveness of penicillin, both on a weight basis and on a molecular basis, in relation to the antibacterial activity of the two most potent quinones is undoubtedly higher than these calculations serve to indicate. The higher amounts of the quinones required for bacterial inhibition in glucose broth in comparison to the quantities needed for the same purpose in plain broth is probably due to the fact that glucose broth is a more favorable medium for the growth of staphylococci.

There is some suggestion in Table I that the minimum antibacterial concentrations of quinones is conditioned in part by the number of organisms in the inoculum. Some additional information as to the magnitude of this factor was obtained in regard to 2-methyl-1,4-naphthoquinone using inocula of 1,500, 11,000 and 147,000 staphylococci. The minimum concentrations of this quinone required to prevent detectable growth of the organisms for 24 hours in broth were respectively 0.50, 1.0 and 1.25 mg per 100 cc.

On account of the growth characteristics

TABLE III.
In vitro Antibacterial Effects Against a Strain of Streptococcus of Quinones Compared on a Molecular and on a Weight Basis.

Compound	Minimum concentrations required for complete: Inhibition of growth: Mols per 100 cc x10⁶	Mg per 100 cc	Sterility: Mols per 100 cc x10⁶	Mg per 100 cc
2-Methyl-1,4-naphthoquinone	1.16	0.200	1.16	0.200
2,6-Dimethoxy-benzoquinone	<0.290	<0.049	<0.29	<0.049
1,4-Naphthoquinone	0.87	0.137	1.15	0.183
Tolu-hydroquinone	0.58	0.072	0.58	0.072
2-Methyl-1-naphthol-4-amino hydrochloride*	1.45	0.303	>5.8	>1.21
2-Methyl-1,4-naphthoquinone sodium bisulfite addition compound†	1.45	0.400	>5.8	>1.60

* "Synkamin" of Parke Davis.
† Abbott's "Hykinone."

of the streptococcus C203, it was necessary to use 8-hour cultures, to terminate the incubation after 16 hours, to employ 0.5% glucose broth, and to use inocula of higher counts. The experiments were otherwise carried out essentially as described above. Sterility tests were done after the incubation period, by inoculating 1 cc of the solutions into blood agar pour plates.

The results in Table III, pertaining to the first 4 substances, were obtained in the course of the experiment using inocula of 27,000 streptococci. Inocula of 153,000 organisms were used in the experiment which gave the data with respect to "Synkamin" and "Hykinone." It will be noted that several of these substances appear to exert a considerably greater antibacterial action against the C203 streptococcus than was observed in the case of the staphylococcus. None of the substances named in Table III in a concentration as high as 29.0×10^{-6} mols per 100 cc exerted any bacteriostatic or bactericidal effect against a strain of *Escherichia coli*.

14263

Production of Hypoprothrombinemia and Hypocoagulability of the Blood with Salicylates.*

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It has recently been reported by Link, Overman, Sullivan, Huebner and Scheel[1] that salicylic acid and sodium salicylate administered repeatedly, or in single doses, orally or intravenously, are capable of producing hypoprothrombinemia in rats. It had already been established[2,3] that salicylic acid is a degradation product of Dicumarol (3,3′ methylenebis (4-hydroxycoumarin)), which is now recognized to be a hypoprothrombinemic agent. In August of 1942 the observations of Link *et al.*[1] were made known to us, and shortly thereafter clinical investigations were begun in an attempt to reproduce the animal experiments. At the same time Dr. Shepard Shapiro of New York City Welfare Hospital, New York University, Division III, began similar studies independently.

The experimental group consisted of 31 adults, males and females, about a fourth of whom were normal individuals—nurses, technicians, and medical students—and the rest patients in the State of Wisconsin General Hospital who were afflicted with a wide variety of illnesses, arthritis of various types being the most common. These subjects received salicylates, in the form of acetylsalicylic acid or sodium salicylate in daily doses ranging from 20 to 80 grains (1.3 g to 5.3 g) for periods of 3 to 11 days.

Methods. The prothrombin time was determined on undiluted plasma with Quick's method as modified by Pohle and Stewart.[4] On each day that tests were made, one or more untreated patients was tested to serve as a control upon the technic used and the potency of the thromboplastin. Ordinarily the thromboplastin was of such potency as to result in a normal prothrombin time of 10 to 11 seconds for this group of experiments. The approximate percentages of prothrombin in human plasma as derived from the chart of Pohle and Stewart are as follows: A time of 12.5 seconds indicates a concentration of

* These studies were aided by a grant from the Wisconsin Alumni Research Foundation.

1 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

2 Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

3 Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 529.

4 Pohle, F. J., and Stewart, J. K., *Am. J. Med. Sc.*, 1939, **198**, 622.

TABLE I.
Effect of Administration of Acetylsalicylic Acid in 8 Individuals upon the Prothrombin Time and Coagulation Time.

	Days Control*	1*	2*	3*	4*	5*	6*	7	8
Prothrombin time in sec, avg	10.4	11.6	11.4	11.9	11.6	11.7	11.9	11.0	10.8
Coagulation time in min, avg	16.6	18.3	17.6	18.1	21.3	18.5	17.7	14.5 (4 cases)	17.6 (5 cases)

* 5 grains (0.3 g) acetylsalicylic acid q.i.d. for seven days.

about 50% of normal; 19 seconds, 25% of normal; and 30 seconds, 12.5% of normal.

The coagulation time was measured at room temperature by the two-tube method of Lee and White.[5] The precautions that were employed to insure accuracy have already been described. The liver function in many but not all was established as normal by the intravenous hippuric acid test of Quick.[6]

Materials. In all but one group of experiments the salicylates were administered orally, in tablet form, in 4 or more equally divided doses. In one series of experiments single doses of 30 to 40 grs. (2 to 2.6 g) were given.

Results. Thirteen subjects received 80 grains (5.3 g) of acetylsalicylic acid daily for periods of 3 to 11 days. For all but one of these both the prothrombin time and coagulation time varied significantly. The increase in prothrombin time ranged from a minimum of 1 second, which is significant under the controlled conditions set up, to a maximum of 4.5 seconds. In most cases the individual maximal prolongation was 2 or 3 seconds, a reduction of prothrombin to about 50% of normal; the coagulation time increased from an average control level of 18 minutes to 25 minutes. Usually an effect was discernible the day after the first administration of the drug, but in most instances the maximal hypoprothrombinemia and hypocoagulability were not observed until after three or four days or more of drug administration. They were not always exactly synchronous in their appearance, although there was, as might be anticipated, some rough parallelism. After administration of the drug had ceased the prothrombin level and coagulability of the blood usually returned to normal in from 2 to 4 days.

Smaller doses of acetylsalicylic acid, 20 grs. (1.3 g), were given to 8 subjects, 7 of whom were normal individuals. In every one of these the prothrombin time increased significantly, and in 7 of the 8 the coagulation time of the blood also increased significantly. The averages for the group are shown in Table I.

An individual experiment of this group is shown in Chart I.

Acetylsalicylic acid was administered in single doses of 30 grains (2 g) to 6 patients and in single doses of 40 grains (2.6 g) to 6

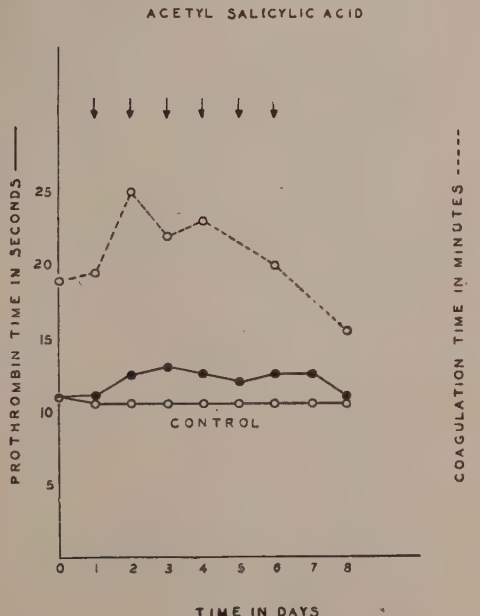

CHART 1.

Effect of administration of 20 grains (1.3 g) of acetylsalicylic acid in 4 divided doses daily to a male patient, T.U. Arrows indicate days that drug was administered.

[5] Lee, R. I., and White, P. D., *Am. J. Med. Sc.*, 1913, **145**, 495.

[6] Quick, A. J., *Am. J. Med. Sci.*, 1933, **185**, 630.

TABLE II.
Effect of Administration of Acetylsalicylic Acid and Vitamin K in 6 Individuals upon the Prothrombin Time and Coagulation Tme.

	Days control P	1 *P	2 *P	3 *P	4 *P	5 *P	6 *P
Prothrombin time, sec, avg	10.9	10.8	11.1	11	11	11.3	11.1
Coagulation time, min, avg	14.5	13.6	14.7	14.6	15.5	14.7	15 4 cases

P—2 mg Proklot 3 times a day.
*P—2 mg Proklot 3 times a day, 2-methyl-1,4-naphthaquinone (Proklot), and 5 grs (0.3 g) acetylsalicylic acid 4 times a day.

others, but no very significant increase in the prothrombin or blood coagulation time resulted. To consistently produce alterations in man with ordinary doses, repeated administration of a salicylate is usually necessary. There is a similarity here to the results in animals.[1]

Sodium salicylate was administered to 6 patients in divided doses of 75 to 80 grs. (5 to 5.3 g) for periods of 7 and 8 days, and in divided doses of 40 grs. (2.6 g) to three others for periods ranging from 3 to 8 days. The effects with the smaller doses were similar to those with the larger and were similar in all respects to those with acetylsalicylic acid. Significant hypothrombinemia and hypocoagulability occurred in 7 of the 9 patients. Prothrombin time increased from a control level of 11 seconds to a maximum of 15 seconds. The minimum prolongation in these 7 patients was 1.0 second. In one patient the coagulation time was increased from 19 to 32 minutes. This great increase was exceptional, but significant prolongation in the coagulation time appeared to be the rule.

Salicylates and Vitamin K. It is now well known that vitamin K will usually correct the hypoprothrombinemia associated with obstructive jaundice or that of the newborn, and that it will fail, save with minimal effective doses of Dicumarol and very large doses of vitamin K,[7] to correct the hypoprothrombinemia resulting from Dicumarol administration. Hence it was a matter of interest to determine whether or not the combined administration of acetylsalicylic acid and vitamin K would prevent the hypoprothrombinemia. It has already been shown by Link *et al.*[1] that vitamin K was completely effective in preventing the salicylate hypoprothrombinemia in animals.

Six subjects, 2 of whom were the same persons used in the study reported in Table I, were given, after control determinations of prothrombin time and coagulation time, 2 mg of 2-methyl-1,4-naphthaquinone (Proklot) three times a day and, beginning a day later, 0.3 g (5 grs.) of acetylsalicylic acid 4 times a day. Both drugs were continued daily for 4 days thereafter. In not one of the 6 individuals did a significant change in the prothrombin time or coagulability of the blood occur. This was particularly striking in the 2 individuals, B.H. and M.S., who participated in the previous study, since in both of them striking changes in prothrombin and in coagulation time had occurred before.

The averages of the results are shown in Table II and they can be compared with Table I.

Discussion. These studies in man confirm the observations of Link, Overman, Sullivan, Huebner, and Scheel.[1] The basis for the investigations lies in the recognition by Huebner[2,3] that Dicumarol, which is capable of producing hypoprothrombinemia, might conceivably be degraded in the body into salicylic acid, and the salicylic acid or a product arising therefrom causes the hypoprothrombinemia.[1] This matter has been discussed in detail in an excellent paper by Link and his associates.[1]

The earlier studies in animals and the observations here recorded explain certain long-known and recorded observations, which have received little or no attention, upon the hazard of hemorrhage that attends the ex-

[7] Shapiro, S., Redish, M. J., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 12.

hibition of large doses of salicylates. Binz[8] stated that in some persons salicylic acid produced hemorrhages in the mucous membranes, and frequent or excessive menstruation in some females. Though the mechanism was not explained, he urged caution. Purpura following the administration of a salicylate was reported by Ramond[9]. Hurst and Lintott[10] reported a case wherein hematemesis attended the administration of aspirin.

It has been widely accepted that epistaxis and other hemorrhagic symptoms, such as hematuria, are not unusual manifestations of acute rheumatic fever. Rinehart, Connor, and Mettier[11] have suggested that a scorbutic state might be the basis of the hemorrhagic manifestations in these patients. This and other readily conceivable factors might furnish the explanation. But the possibility still remains that in some of these cases the administration of large doses of salicylates, a very common practice, might be responsible. Pertinent to these considerations, however, are the reports of Daniels and Everson,[12] Samuels, Ritz and Poyet[13] and Ritz, Samuels and Addiss[14] on the increased excretion of vitamin C in human beings and animals, and the tissue depletion of ascorbic acid in animals following the administration of salicylates.

Despite the observations here recorded, one is not justified in concluding that salicylate administration is a common or important cause of hemorrhage in human beings. Salicylates are used widely in large doses in many types of arthritis and other conditions, with and without medical supervision, without hemorrhage being anything but a rare and insignificant complication.

Several attractive hypotheses might suggest themselves to the reader to explain the mechanism whereby salicylates produce hypoprothrombinemia and hypocoagulability of the blood. For the present, however, it seems wiser to withhold speculation since the mode of operation is not understood.

Conclusions. 1. The oral administration of acetylsalicylic acid and sodium salicylate to human beings in daily doses of 20 to 80 grains (1.3 to 5.3 g) consistently produced hypoprothrombinemia and hypocoagulability of the blood. 2. The administration of vitamin K with the salicylate prevented the development of hypoprothrombinemia and prolongation of the coagulation time. 3. It is theoretically possible that the not unusual hemorrhagic manifestations of acute rheumatic fever may be due in some cases, at least in part, to the large doses of salicylates so commonly administered.

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8 Binz, C., *Vorlesungen über Pharmakologie,* 2nd ed., Berlin, 1891; *Berlin Klin. Wchnschr.,* 1893, **30**, 985.

9 Ramond, *Le Progres Med.*, 1904, **33**, 471.

10 Hurst, A. Sir, and Lintott, G. A. M., *Guy's Hosp. Rep.*, 1939, **89**, 173.

11 Rinehart, J. F., Connor, C. L., and Mettier, S. R., *J. Exp. Med.*, 1934, **59**, 97.

12 Daniels, A. L., and Everson, G. J., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 20.

13 Samuels, L. T., Ritz, N. D., and Poyet, E. B., *J. Pharm. and Exp. Therap.*, 1940, **68**, 465.

14 Ritz, N. D., Samuels, L. T., and Addiss, G., *J. Pharm. and Exp. Therap.*, 1940, **70**, 362.

14264

Relationship of Bactericidal Potency to Length of Fatty Acid Radical of Certain Quaternary Ammonium Derivatives.

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From Emulsol Corporation Research Laboratory.

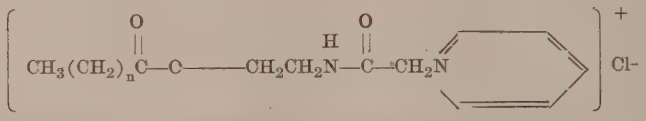
Within recent years, a number of quaternary ammonium derivatives have been introduced as antiseptic substances. These compounds are characterized in that they have a long chain lypophile group in the cation balanced with a hydrophile group as the anion. Some of these compounds have predominantly long chain lypophile alkyl groups attached directly to nitrogen.[1,2] Others have a long chain lypophile group interrupted by ether linkages or phenyl groups.[3,4]

Recently new types of quaternary ammonium cationic compounds have been introduced as bactericidal agents in which the lypophile group is interrupted by carboxyl and amide linkages.[5] They are characterized by the fact that in the cationic part of the molecule there is an acyl group, a colamine group and a formyl-methyl quaternary ammonium radical.

The introduction of the carboxyl and amide groups into the molecule has an effect upon the functional properties of the compound. A study was made of the effect on the bactericidal value of these compounds in relation to the chain length of the fatty acid radical which enters in the lypophile group, and their respective relations to detergency. The compounds were prepared according to the method outlined by Katzman.[6] The fatty acids were varied from C_8 to C_{18} and the bactericidal value of the homologous compounds was determined according to the official F.D.A.[7] Method. The results are tabulated in Table I in which it is shown that varying the fatty acid radical has a decided

TABLE I.

Effect of Changing the Fatty Acid Radical on Killing Power of



No. of carbons in $CH_3(CH_2)_nC(=O)O-$	*Average maximum dilution to kill organism in 10 minutes			
	20°C		37°C	
	S. aureus	*E. typhosa*	*S. aureus*	*E. typhosa*
8	1: 350	1: 550	1: 800	1: 1,300
10	1: 2,750	1: 2,000	1: 3,500	1: 4,000
12	1:14,000	1:10,000	1:25,000	1:30,000
14	1:30,000	1:30,000	1:55,000	1:55,000
16	1: 7,000	1: 3,500	1: 9,000	1:11,000
18	1: 4,000	1: 1,500	1: 7,000	1: 6,000

* According to F.D.A. official method.

1 Domagk, G., U. S. Patent No. 2,108,765, 1938.

2 Domagk, G., *Deut. Med. Wochenschr.*, 1935, **21**, 829.

3 Bruson, H. A., U. S. Patent No. 2,115,250, 1938.

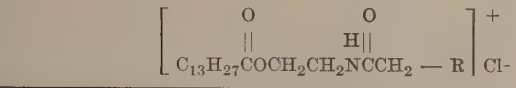
4 Rawlins, A. L., Sweet, L. A., and Joslyn, D. A., *J. Am. Pharm. Assn.*, 1943, **32**, 111.

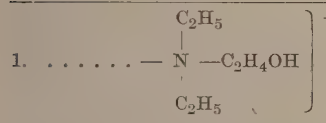
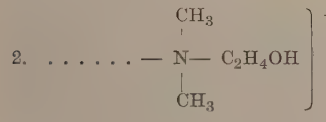
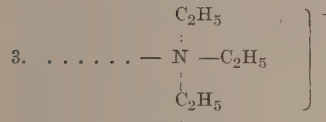
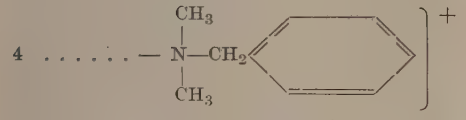
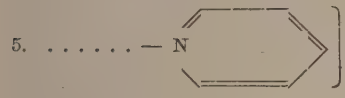
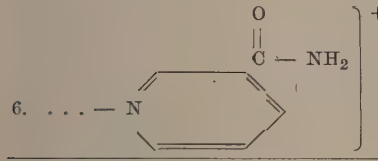
5 Epstein, A. K., and Harris, B. R., U. S. Patent No. 2,290,173, 1942.

6 Katzman, M., U. S. Patent No. 2,189,664, 1940.

7 Ruehle, G., and Brewer, C. M., 1931, U. S. Food and Drug Administration Method of Testing Antiseptics and Disinfectants, Circular No. 198, U. S. D. A.

TABLE II.
Effect of Changes in R on Killing Power of



	R	Dilution required to kill organisms in 10 minutes at 20°C	
		Staph. aureus ×1000	*E. typhosa* ×1000
1.		1:30	1:30
2.		1:30	1:30
3.		1:25	1:30
4		1:25	1:30
5.		1:30	1:30
6.		1:10	1:15

effect on the bactericidal potency.

Kuhn and Dann[8] have shown, in studying the bactericidal effects of alkyl dimethyl sulphonium iodides on *B. coli* and *Staphylococcus aureus,* and varying the alkyl group from methyl to butyl, octyl, lauryl and cetyl, that the optimum bactericidal function for coli was obtained with the lauryl derivative, and for Staphylococci, the cetyl radical was most effective.

Kuhn, Jerchel and Westphal[9] reported on the series di-alkyl methyl benzyl ammonium chloride varying the alkyl radicals from C_4 to C_6, C_8, C_{12} and C_{16}, respectively. They found optimum potency at C_8 for Staphylococci, diphtheria, *B. coli,* Friedländer, but for paratyphoid, C_8 and C_{12} radicals were more effective. The same authors reported that in using C_8, C_{12} and C_{16} radicals in the alkyl groups of alkyl dimethyl benzyl ammonium chloride, the greatest bactericidal potency was obtained from the compound containing C_{16} in the alkyl chain. In the specific homologous series of the quaternary compounds which we studied, we found that in varying the fatty acids from C_8 to C_{18} re-

[8] Kuhn, R., and Dann, O., *Ber.*, 1940, **73**B, 1092.

[9] Kuhn, R., Jerchel, D., Westphal, O., Moeller, E. F., and v. Czernucki-Hrebeljanowitsch, M., *Ber.*, 1940, **73**, 1095.

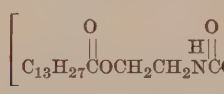
spectively, the optimum bactericidal function was obtained when the compound contained the fatty acid radical of C_{14} and that the compound which contained the C_8 radical had the lowest bactericidal value.

It is therefore evident that the bactericidal functions of these types of compounds depend upon the specific structure and the configuration of the molecule and also to a certain extent on the type of organism which is used in the test.

A compound designated as E 607 (Special) of the same series as shown in Table II was prepared from a mixture of C_{12} and C_{14} fatty acids and tested bacteriologically against *Staph. aureus* and *E. typhosa* at 20°C and 37°C respectively. The results show that the addition of C_{14} fatty acid ester increased the bactericidal potency of the C_{12} compound almost twofold.

One member of the series in Table I was selected for study of the effect of varying the groups attached to the pentavalent nitrogen on the bactericidal potency. For the acyl radical, C_{14} was selected as the fatty acid ester which gave the highest results. The compounds prepared are tabulated in Table II together with their bactericidal activity against *E. typhosa* and *Staph. aureus*.

It is noteworthy that there is little relative difference in the bactericidal potency of the quaternary ammonium derivatives in the series of



when the R radical is varied by substituting three lower molecular weight groups on the pentavalent nitrogen as shown in examples one, two, three, four and five respectively in Table II. If, however, R is nicotinic amide as shown in example No. 6 in Table II the bactericidal activity is reduced considerably. If compounds No. 5 and No. 6 in Table II are compared it appears that the substitution of an amide group in the beta position of the pyridine ring of compound No. 5 reduces the bactericidal activity of the compound. This is parallel to the vast reduction in the toxicity of pyridine when its beta hydrogen is substituted with $-C(=O)NH_2$ group to form nicotinic amide.

The phenol coefficient as determined by the standard F.D.A. method[7] may not be accepted in all laboratories as proof of the germicidal value of these compounds. The toxicity indices were therefore determined according to the technics of Welch and Brewer [10, 11] and Hirsch and Novak.[12]

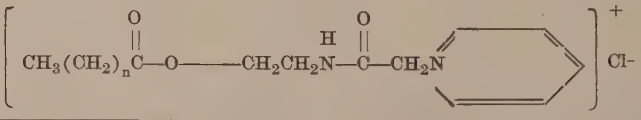
The lauric acid ester of colamino formyl methyl pyridinium chloride (which was designated as E 607) gave a toxicity index of less than .6 by both of these technics. A toxicity index which is less than unity is considered safe for tissue antisepsis.

Summary. (1) A number of homologs of quaternary ammonium derivatives were prepared of the series (see formula below) varying the carbon chain in the fatty acid radical from C_8 to C_{18} and the bactericidal potency of each compound was determined using *Staph. aureus* and *E. typhosa* as test organisms.

(2) In this series it was found that the C_{14} homolog possesses the maximum bactericidal activity and the sequence of diminishing bactericidal power is as follows:

$$C_{14}>C_{12}>C_{16}>C_{18}>C_{10}>C_8.$$

(3) A number of myristic acid esters of colamino formyl methyl quaternary ammonium chloride was prepared wherein the molecule as a whole remained constant in its structural configuration except as to the lower molecular weight substitution groups on the pentavalent nitrogen. The bactericidal ac-



[10] Welch, H., and Brewer, C. M., *J. Immunol.*, 1942, **43**, 25.

[11] Welch, H., Hunter, A. C., and Slocum, G. G., *J. Lab. and Clin. Med.*, 1942, **27**, 1432.

[12] Hirsch, M. M., and Novak, M. V., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 376.

tivity of this series of compounds was determined using the same test organisms and was found that there is no appreciable difference in the respective compounds with the exception of the nicotinic amide derivative.

(4) The substitution of beta hydrogen in the pyridine ring by $-\overset{\overset{O}{\|}}{C}NH_2$ reduces the bactericidal potency of the pyridinium derivative.

14265

Taxonomic Relationships in the Genus Proteus.

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Recently Cope and Kilander[1] described 83 strains isolated from gastroenteritis patients, contacts and foodhandlers with suggestive or definite history of enteric disorders as "atypical enteric organisms of the *Shigella* group." Stuart *et al.*[2] reported 19 comparable strains tentatively classed as "anaerogenic paracolon" cultures which were isolated in this laboratory or received from the laboratories of several states. To date the number of strains studied by us has increased to 48, including 5 strains kindly furnished by Dr. E. J. Cope. Forty-three of the strains were isolated from fecal specimens of gastroenteritis patients or foodhandlers, one from the blood of a patient and 4 from apparently normal contacts. To facilitate discussion the cultures studied in this laboratory will be called, type 33111.

Cultural Characteristics. Type 33111 cultures grew readily on eosin methylene blue agar and on Salmonella Shigella agar forming transparent and colorless colonies. All strains isolated in this laboratory were motile in semisolid, tryptone agar (0.25% agar) at 25°C. Several strains received from different laboratories as nonmotile on isolation were motile under the conditions specified. The motility of type 33111 cultures was usually poor and sometimes negative at 37°C. On agar slants an occasional strain showed slight spreading. On nutrient agar plates (1.0% agar) inoculated by touching the center of the plate with a needle, the growth of some strains covered the entire surface of the plate, others partially, others spread slightly while a few showed no tendency to spread after 48 hours at 25°C. The swarming characteristic of some strains was suggestive of the genus *Proteus* and flagella stains were made on representative strains of each species in this genus. *Pr. vulgaris, Pr. mirabilis, Pr. morganii* and type 33111 cultures showed peritrichous flagellation. *Pr. hydrophilus* and *Pr. ichthyosmius* possessed a single polar flagellum. Kulp and Borden[3] and Guthrie and Hitchner[4] found *Pr. hydrophilus* to be monotrichate.

Biochemical reactions. Type 33111 cultures fermented glucose and mannitol in 24 to 48 hours and sucrose in 14 to 21 days. Nineteen strains fermented salicin in 24 to 48 hours. Lactose and maltose were not attacked. Six strains produced a bubble to 10% gas in glucose and mannitol and one of these a bubble of gas in salicin but no gas was produced in sucrose by any culture. The remaining strains were anaerogenic in all fermentable carbohydrates.

Using a new urea medium Rustigian and Stuart[5] showed that the production of urease by *Pr. vulgaris* and *Pr. mirabilis* could be de-

1 Cope, J. E., and Kilander, K., *Am. J. Pub. Health*, 1942, **32**, 352.

2 Stuart, C. A., Wheeler, K. M., Rustigian, R., and Zimmerman, A., *J. Bact.*, 1943, **45**, 101.

3 Kulp, W. L., and Borden, D. G., *J. Bact.*, 1942, **44**, 673.

4 Guthrie, R., and Hitchner, E. R., *J. Bact.*, 1943, **45**, 52.

5 Rustigian, R., and Stuart, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 108.

tected in 4 to 5 hours while strong reactions were obtained in 10 to 12 hours. *Pr. morganii* attacked urea strongly but more slowly. To further study urea decomposition as a limiting characteristic for *Proteus* 689 cultures representing all genera of the family *Enterobacteriaceae* have been tested. None of 13 *Serratia*, 24 *Erwinia*, 40 coliform intermediates, 32 *Salmonella* and 33 *Shigella* cultures attacked urea. Of 217 *Escherichia*, 169 paracolon and 161 *Aerobacter* cultures 4, 15 and 51 strains respectively gave weakly positive reactions, pH 7.0 to 7.2 in 5 days. Forty-seven of the 48 type 33111 cultures decomposed urea rapidly and strongly. One urea negative strain of *Proteus* has been reported.[5] All type 33111 cultures produced indole and grew on citrate agar in 24 hours. Gelatin was not liquefied and neither hydrogen sulfide nor acetyl-methyl-carbinol was produced by any strain.

Except for 2 strains Wood *et al.*[6] found that type 33111 cultures possessed the ability to reduce trimethylamine oxide and it was found in this laboratory that these cultures were incapable of growing in glucose broth at 45° C.

Serological Reactions. The antigenic relationships of type 33111 strains to one another is not clear. The fairly good antigenic homogeneity of the first 19 cultures studied[2] was not maintained as additional strains were isolated. In the first antiserum prepared against a type 33111 culture several strains which upon isolation agglutinated to titer and completely adsorbed or markedly reduced the homologous titer later either failed to agglutinate or did not reduce the titer of the same antiserum upon adsorption. Moreover cultures not agglutinating in this antiserum but capable of markedly reducing the titer for the homologous strain were encountered. A similar condition has been observed for *Proteus* in this laboratory[7] and by Taylor.[8] Agglutination and adsorption tests with additional strains and antiserums showed type 33111 cultures to be antigenically heterogeneous.

Selected type 33111 strains were tested in pooled *Shigella paradysenteriae*, in *Sh. sonnei, Sh. alkalescens* and 12 *Proteus* antiserums. Negative reactions were obtained in the *Shigella* antiserums. Three cultures agglutinated to 640 in one *Pr. vulgaris* antiserum with a homologous titer of 20480 and to 1280 in one *Pr. mirabilis* antiserum with a homologous titer of 20480. Fourteen cultures agglutinated to 160 to 320 in 3 *Pr. morganii* antiserums (homologous titers 20480 to 40960). The reactions in each instance were due to flagellar antigens.

Because of their cultural, biochemical and serological reactions Rustigian and Stuart[9] suggested that type 33111 cultures be tentatively called, *Proteus* entericus.

Recently Wood and Baird[10] found that the less restricted *Shigella* species, *sonnei, alkalescens, dispar* and *ceylonensis* were able to reduce trimethylamine oxide. Stuart and Rustigian[11] found that these same species grew readily and 97% of 190 cultures tested produced a strong acid reaction in glucose broth at 45°C. A culture of *Sh. rettgeri*, however, reduced trimethylamine oxide but failed to grow at 45°C. A review of the literature revealed that St. John-Brooks and Rhodes[12] found *Sh. rettgeri* indole positive and Edwards[13] found this organism to be actively motile under suitable conditions. The biochemical and IMViC reactions of Dr. Wood's strain and of the A. T. C. C strain of *Sh. rettgeri* were identical with those of type 33111 cultures, including rapid and strong hydrolysis of urea. Furthermore the 2 strains of *Sh. rettgeri* agglutinated but not to titer in 2 of 3 Type 33111 antiserums.

Discussion. Neter[14] suggested that *Sh.*

6 Wood, A. J., Baird, E. A., and Keeping, F. E., *Can. J. Res.*, in press.

7 Rustigian, R., and Stuart, C. A., manuscript in preparation.

8 Taylor, J. F., *J. Path. and Bact.*, 1928, **31**, 897.

9 Rustigian, R., and Stuart, C. A., *J. Bact.*, 1943, **45**, 198.

10 Wood, A. J., and Baird, E. A., *J. Bact.*, in press.

11 Stuart, C. A., and Rustigian, R., *J. Bact.*, in press.

12 St. John-Brooks, R., and Rhodes, M., *J. Path. and Bact.*, 1923, **26**, 433.

13 Edwards, P. R., personal communication.

14 Neter, E., *Bact. Rev.*, 1942, **6**, 1.

rettgeri should be eliminated from the genus *Shigella*. In our opinion *Sh. rettgeri* and type 33111 cultures are the same organism and should be redescribed as *Proteus rettgeri* in the genus *Proteus* or in the appendix of that genus. Because the majority of *Pr. rettgeri* strains were anaerogenic and all strains fermented mannitol some investigators will question the feasibility of placing this organism in the *Proteus* group. *Pr. vulgaris*, *Pr. mirabilis* and *Pr. morganii* produce small gas volumes and old cultures of *Pr. vulgaris*[7,15] not infrequently become anaerogenic and since in the present study some of the strains of *Pr. rettgeri* were aerogenic actual gas production could be considered a flexible criterion for *Proteus*. Despite the fact that Topley and Wilson[16] and St. John-Brooks and Rhodes[17] consider the inability to ferment mannitol a genus characteristic it would seem that ability to attack urea and to swarm are more important genus characteristics. The cultural, biochemical and serological reactions of *Pr. rettgeri* illustrate the difficulty in drawing strict lines of division between related groups of bacteria.

Pr. rettgeri has been described vicariously as a *Bacterium*,[18] *Bacillus*,[12] *Eberthella*,[19] and *Shigella*.[20] This sequence of events in our opinion was not due to carelessness but was brought about by increases in the delicacy and particularly in the number of tests used to differentiate species of bacteria since the isolation of *Pr. rettgeri* in 1909. It seems strange, however, that this relatively rare organism originally isolated from a cholera-like epidemic among fowls should have been isolated from more than 100 gastroenteritis patients or foodhandlers in the past 3 or 4 years.

The taxonomic position of *Pr. hydrophilus* and *Pr. ichthyosmius* has already been questioned.[4,5,17,21] These organisms do not decompose urea, are monotrichate, do not swarm and some strains produce from 20 to 100% gas volumes at 25°C from fermentable sugars, and produce acid and gas slowly from lactose at this temperature. A culture of *Pr. pseudovaleriei* received from Dr. de Assis of Brazil while peritrichate did not attack urea, produced approximately 30% gas in fermentable carbohydrates and appeared to possess all the characteristics of a paracolon organism. For these reasons it is recommended that *Pr. hydrophilus*, *Pr. ichthyosmius* and *Pr. pseudovaleriei* be dropped from the genus *Proteus*.

15 Welch, H., and Poole, A. K., *J. Bact.*, 1934, **28**, 523.

16 Topley, W. W. C., and Wilson, G. S., *The Principles of Bacteriology and Immunity*, 1936, second edition.

17 St. John-Brooks, R., and Rhodes, M., Third International Congress for Microbiology, Report of Proceedings, 1939, 167.

18 Hadley, P., Elkins, M. W., and Caldwell, D. W., Agr. Exp. Sta. Rhode Island State College, 1918, Bull. No. 174.

19 Bergey, *et al.*, *Manual*, 1st ed., 1923, 232.

20 Bergey, *et al.*, *Manual*, 5th ed., 1939, 476.

21 Reed, G. B., and Toner, C. G., *Can. J. Res.*, 1941, **19** D, 139.

14266

Effect of Temperature on Bacteriostatic Action of Sulfathiazole and Other Drugs. I. *Escherichia coli*.

S. W. LEE AND E. J. FOLEY. (Introduced by Marion B. Sulzberger.)

From the Wallace Laboratories, Inc., New Brunswick, N.J.

As a possible means of throwing light on the mode of action of the sulfonamides, the bacteriostatic effect of sulfathiazole, alone and with other drugs, has been studied as a function of temperature. Other such studies have given revealing information concerning the interaction of these substances with luminous bacteria, as well as isolated enzyme systems.[1]

1 Johnson, Frank H., *Science*, 1942, **95**, 104.

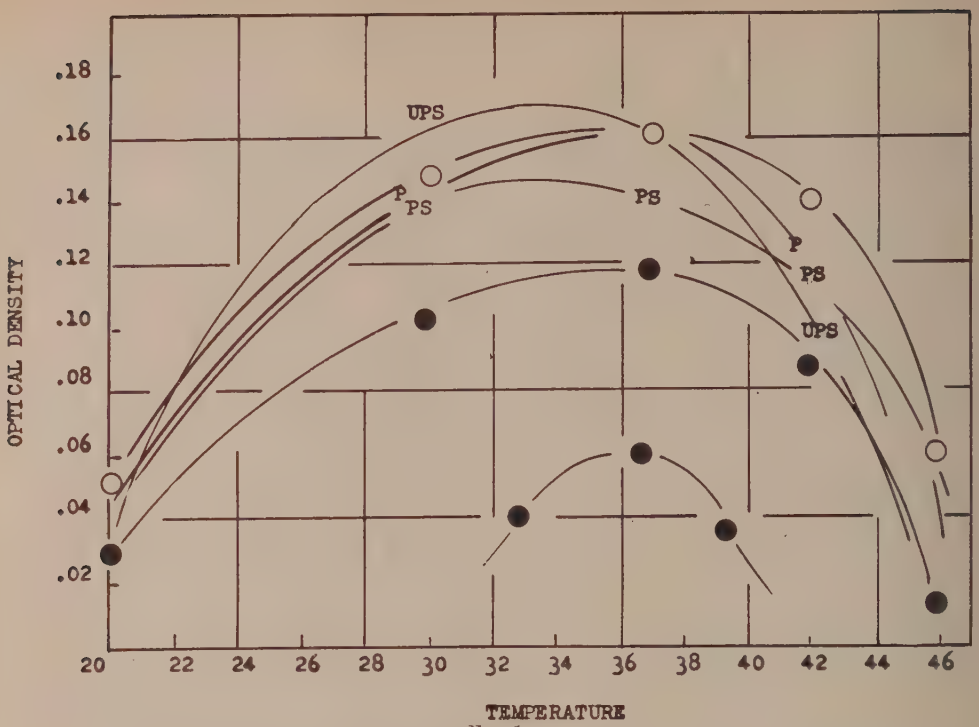

FIG. 1.

The Effect of Various Substances on the Rate of Growth of *E. coli*, as a Function of Temperature.

Open circles: Organism in medium SG (controls). Constant inoculum in all cases of about 300 million organisms.

Solid circles: Organism in medium SG and 10–4 M sulfathiazole.

P: Organism in SG and *p*-aminobenzoic acid 10–5 M.

PS: Organism in SG, PAB 10–5 M and sulfathiazole 10–4 M.

UPS: Organism in SG, urea 1.5%, PAB 10–5 M and sulfathiazole 10–4 M.

Readings taken after six hours.

Solid circles, lowest curve. Organism in medium SG and 10–4 sulfathiazole. Inoculum, about 30 million. Control for this curve same as for the remainder.

With the work of Kohn and Harris[2] as a guide, several experiments have been carried out on *Escherichia coli*. This organism was chosen because of its susceptibility to the drugs in question, and because it grows well over a rather wide temperature range. Included in this report are data concerning the rate of growth of *E. coli*, as a function of temperature, and to some extent, of the concentration of the added rate-influencing substances. The substances chosen are among those of current interest, sulfathiazole, *p*-aminobenzoic acid and urea.[3] These have been studied separately, and in some of the possible combinations. In general, the first results are in line with what one would predict.

In the method chosen, massive inocula of *E. coli* were added to the water clear SG (salt and glucose) medium,[2] or to this medium containing the indicated quantities of added substances. The large inocula were chosen to give, in a day's time, large turbidity differences, which were measured with a

[2] Kohn and Harris, *J. Pharm. and Exp. Therap.*, 1943, **77**, 1.

[3] Tsuchiya, Tenenberg, Clark and Strakosch, PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

Coleman Universal spectrophotometer with the wave length dial set at 600 mμ. Comparisons were made against a SG medium blank, which had received the same treatment as the other tubes, though this always remained perfectly clear. This turbidity measurement, of course, gives the total number of organisms present, without regard to their viability.

A composite graph of results in Fig. 1 shows the effect of various substances and combinations of these substances on the growth rate of *E. coli*, as a function of temperature. It will be noticed that the diminution of the growth rate, as effected by sulfathiazole, increases markedly with temperature. This inhibition amounts to 45% at 42°C (108°F), and only to 24% at 37°C. This is in accord with the work of Bang[4] on gonococcus, and of others. The *p*-aminobenzoic acid, in the concentration used, slightly diminished the growth, except at 37°. This concentration, however, overcomes most of the ill effects of sulfathiazole up to about 37°. At this, and higher temperatures, there is an appreciable inhibition of growth, due to the sulfathiazole, or in part, to the *p*-aminobenzoic acid itself. It is also of importance that *p*-aminobenzoic acid diminished the growth rate at temperatures higher than 37° in a manner which increases with temperature. This would suggest that *p*-aminobenzoic acid exerts a sulfonamide-like effect, provided the temperature is slightly raised. Urea alone, in concentrations up to 2%, acts to increase growth except at temperatures higher than the optimum. This increase amounts to about 10% at 37, and thus the effect of the mixture of *p*-aminobenzoic acid, urea and sulfathiazole (UPS) is accounted for. At slightly higher temperatures, urea alone reduces the growth rate sharply. Thus it might be said that at these temperatures, urea would certainly act conjointly with sulfathiazole in diminishing bacterial growth, at least in the case of *E. coli*. The large role which the inoculum size plays in determining the growth rate is brought out by a comparison of the 2 lowest curves, in which the inoculum is varied by a factor of 10.

In summary, the effect of temperature on the bacteriostatic action of sulfathiazole, *p*-aminobenzoic acid and urea, alone and in several combinations, has been studied on *E. coli*. The growth inhibition increases with temperature for all the substances. It is of importance that *p*-aminobenzoic acid inhibits growth at temperatures higher than 37°, and that at these elevated temperatures it is incapable of showing marked antisulfonamide properties.

4 Bang, Frederik B., *J. Urology*, 1942, **47**, 299.

14267

Temperature and the Bacteriostatic Action of Sulfathiazole and Other Drugs. II. *Streptococcus pyogenes.*

S. W. LEE, JEANNE A. EPSTEIN, AND E. J. FOLEY. (Introduced by Marion B. Sulzberger.)

From the Wallace Laboratories, Inc., New Brunswick, N.J.

Preliminary work carried out on *E. coli* in this laboratory has shown that a study of bacterial growth rates, as influenced by sulfonamides and other drugs at various temperatures, gives interesting information concerning the bacteriostatic action of these substances.[1] In order to extend these observations and to study the generality of the actions, similar experiments have been carried out on *Streptococcus pyogenes*. (Ameri-

1 Lee, S. W., and Foley, E. J., to be published. See companion article.

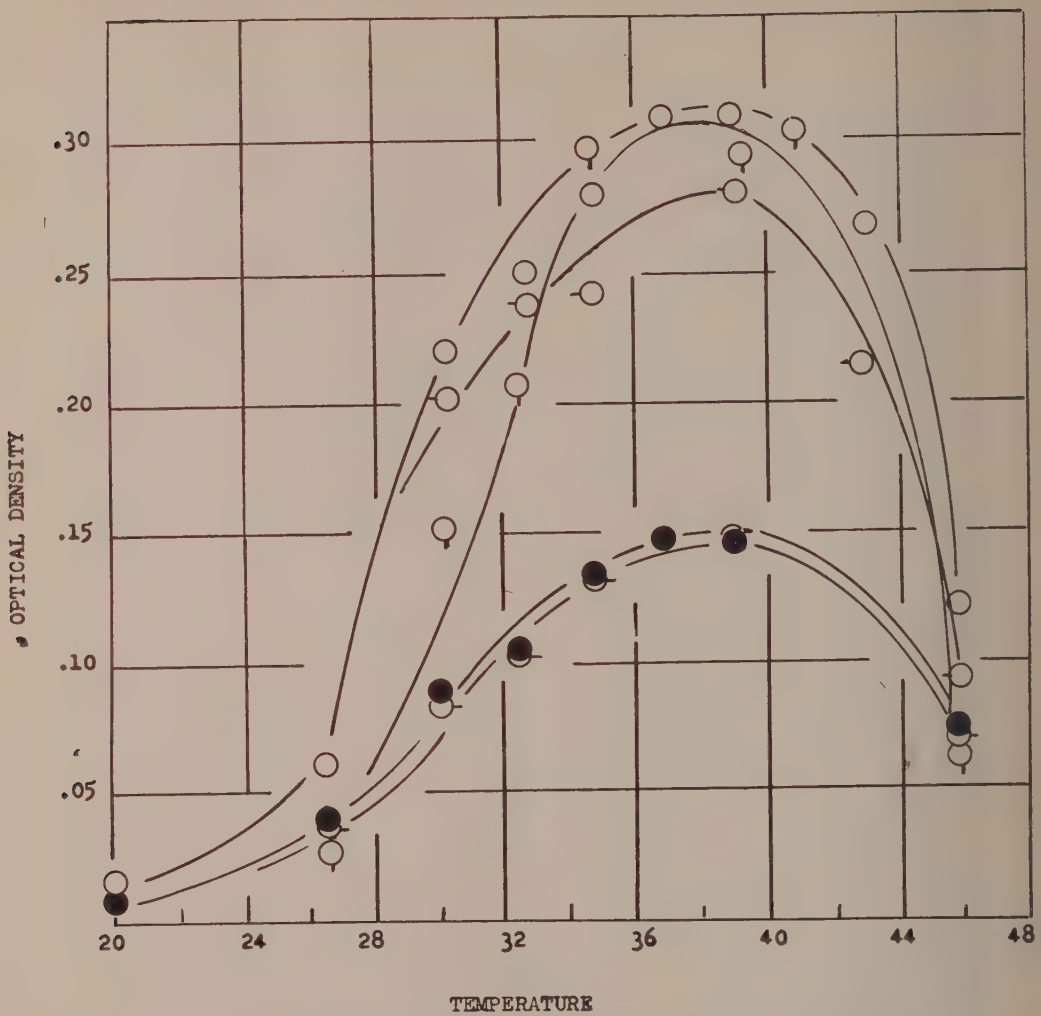

FIG. 1.

The Effect of Various Substances on the Growth Rate of *Streptococcus pyogenes*, as a Function of Temperature.

○ Control, no drug.
○ *p*-aminobenzoic acid 10–5 M, and sulfathiazole 10–4 M.
○ Urea, 1%.
● Sulfathiazole, 10–4 M.
○ Sulfathiazole, 10–4 M and urea, 1%.

can Type Culture Collection number 8668).

The methods used in this series of experiments are essentially those of the earlier work.[1] Inocula which gave an initial population of about 50 million organisms per ml were added to the simplest medium which could be found to support active and rapid growth. This procedure has been advocated as a valid method for studying the action of the sulfonamides.[2] The medium used consisted of 75 parts of the basal medium of

[2] Bell, P. H., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, 1942, **64**, 2905.

Kirby and Rantz[3] and 5 parts of Leventhal blood filtrate, prepared from equal parts of the above basal medium and horseblood by boiling and filtering through cheese cloth and a Seitz funnel. This medium was also found to be relatively free of sulfonamide inhibitors, using as a guide the action of low concentrations (10^{-5} and 10^{-6} M) of sulfathiazole on *E. coli*. The growth measurements were taken at times during which the increase in population was approximately linear (period of retarded growth) as determined by separate experiments. With the inocula used, this time was usually from 4 to 6 hours. The drugs chosen for study were again sulfathiazole, *p*-aminobenzoic acid and urea. Urea is of interest as a potentiator of the sulfonamides. In kind, the results paralleled those obtained on *E. coli*.

Fig. 1 shows most of the experimental results. Optical densities, as obtained from the drum of a Coleman Universal Spectrophotometer, are plotted against temperature. In the case of this organism, the density is directly proportional to population, and a density of 0.20 corresponds to a population of about 2.7 billion organisms per ml. The action of *p*-aminobenzoic acid alone has not been plotted, but at a concentration of 10^{-5} M it increased the growth rate up to the optimum growth temperature, and at higher temperatures exerted a marked inhibition. From the figure, it is clear that up to about 40°, *p*-aminobenzoic overcomes most of the bacteriostatic effects of sulfathiazole. At higher temperatures, the bacteriostatic action of this pair becomes increasingly pronounced. Again, the sulfathiazole shows greater bacteriostatic activity at the higher temperatures. Urea alone greatly diminishes growth at elevated temperatures, but, with this method of study, shows no synergistic action with sulfathiazole, except at the highest temperature.

Summary. The effect of sulfathiazole, *p*-aminobenzoic acid and urea, separately and in combinations, on the growth rate of *Streptococcus pyogenes* has been studied as a function of temperature. The bacteriostatic action of all these substances has been shown to increase with temperature. The sulfonamide-like action of *p*-aminobenzoic acid at higher temperatures is considered to be of particular significance. With the method used, urea shows no synergistic action with sulfathiazole on this organism. These results with *Streptococcus pyogenes* have been found to be analogous to those obtained with *E. coli*.

[3] Kirby, W. M., and Rantz, L. A., *J. Exp. Med.*, 1943, **77**, 29.

14268 P

An Antibiotic Substance Produced by Submerged Cultivation of *Aspergillus flavus*.

CLARA M. MCKEE AND HAROLD B. MACPHILLAMY. (Introduced by Geoffrey Rake.)

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The strain of *Aspergillus flavus* used by White[1] and by Jones, Rake and Hamre[2] for the production of aspergillic acid has been grown in submerged culture with the production of an entirely different antibiotic substance. Erlenmeyer flasks containing a basic Czapek-Dox medium with the addition of adjuvant substances were inoculated with a heavy spore suspension of the mold. The flasks were subjected to constant, moderate agitation at a temperature of approximately 25°C. Within 48 hours there was heavy mycelial growth, and as this progressed, the

[1] White, E. C., *Science*, 1940, **92**, 127.

[2] Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, 1943, **45**, 461.

medium became increasingly alkaline and the antibiotic substance began to appear. Maximal activity, which coincided with a pH of about 8.4, appeared from 7 to 14 days after inoculation, depending upon the medium and method of cultivation.

The ability of this culture of *Aspergillus flavus* to produce an antibiotic substance different from aspergillic acid was not due to a change in the organism since cultures which produced this substance in submerged culture have been shown to produce aspergillic acid again in a typical manner under suitable conditions of surface growth.

The biological characteristics of this antibiotic substance, as so far demonstrated, suggest that it is either penicillin or a very similar substance. Both substances are highly active *in vitro* against gram positive cocci and are relatively inactive against gram negative bacilli. *In vivo* studies in mice reveal that the therapeutic efficacy of the two substances is the same when comparable doses, as determined by titration *in vitro*, are given. This is illustrated in Table I.

Other similarities between the two substances lie in the fact that both are excreted by the kidneys at about the same rate, and both are relatively non-toxic. A true estimate of comparative toxicity, however, can only be attained when purer preparations of the new substance than are now available are obtained. Further likenesses are shown by the fact that cultures which have been rendered resistant to one of the substances are resistant also to the other, although not to other antibiotic substances, and that a bacterial enzyme obtained from a culture of *Staphylococcus aureus* destroys the activity of both substances while it is inactive against aspergillic acid, gliotoxin and gramicidin.

Chemical studies have shown that, in regard to solubility and stability under various conditions, the active agent behaves much like penicillin. It is rapidly inactivated at room temperature below pH 4, and somewhat more slowly above pH 8. It can be extracted from ice-cold aqueous solutions at pH 2 with ether, amyl acetate or chloroform, recovered from the organic solvent with pH 6.5 phosphate buffer, and transformed into a relatively stable sodium salt in the same manner as penicillin. The active agent is also amenable to further purification by the chromatographic methods which have been applied successfully to the fractionation of crude penicillin.

A sodium salt, assaying 240 F.U./mg, which was obtained by chromatographing, showed the following analytical composition: C, 45.36%; H, 4.16%; N, 3.02%; Na, 13.36%. The specific rotation $[\alpha]_D$ was $+108°$ (in water). These data, especially the comparatively high dextrorotation, are not incompatible with the assumption that penicillin or a very similar substance was a constituent of the mixture. Some α-furoic acid, identified by its melting point (129°) and by analysis, was later isolated from this fraction. The presence of this substance would account roughly for the deviation of the analytical figures from those found for penicillin sodium salts in the range of from 500 to 1000 Florey units.

Summary. An antibiotic substance entirely unlike aspergillic acid has been produced by submerged cultivation of a strain of *Aspergillus flavus*. This substance closely resembles penicillin biologically and chemically.

TABLE I.
Comparative Protection of Mice with Penicillin and the Antibiotic Substance from *Aspergillus flavus* Against a Type I Pneumococcus Infection.

Dose	Penicillin No. of mice L.	D.	Antibiotic substance from *Aspergillus flavus* No. of mice L.	D.	Control No. of mice L.	D.
100 Florey Units	9	1	10	0		
10 " "	8	2	7	3		
1 " "	4	6	8	2		
—					0	10

The mice were infected with 1 cc of a 10^{-7} culture.

14269

Vitamin Requirements of *Torula cremoris*.*

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From the Department of Bacteriology and Parasitology, University of Chicago.

In the course of studies dealing with vitamin deficiencies of microörganisms it was found that a laboratory culture of *Torula cremoris* was unable to grow in a simple medium of inorganic salts, ammonium phosphate and glucose. On substitution of a mixture of 19 amino acids for the ammonium phosphate the torula likewise failed to grow and these negative results were in sharp contrast to the luxuriant growth in dextrose broth or agar.

When a mixture of 16 accessory growth factors and related compounds was added either to the amino acid or the ammonium phosphate basal medium growth of this strain of *Torula cremoris* occurred promptly with marked turbidity in 24 hours.

Upon successive elimination of accessory factors from the mixture it was found that 4 of them were necessary for rapid and luxuriant growth. These were nicotinamide (or nicotinic acid), biotin, pantothenic acid and thiamin. When these were supplied the addition of other factors, which were in the original accessory mixture, did not appreciably increase the rate or luxuriance of growth.

The effect upon growth of various combinations of the above 4 factors was next studied. For these experiments biotin was supplied as the methyl ester in a concentration of 0.002 μg per ml, calcium pantothenate, thiamin and nicotinamide in concentrations of 0.2 μg per ml. The ammonium phosphate basal medium was composed of $(NH_4)_2HPO_4$ 0.2%, KH_2PO_4 0.15%, NaCl 0.5%, $MgSO_4$ 0.01% and glucose 0.5%. The amino acid basal medium consisted of 17 amino acids in amounts of 50 to 200 mg each per liter and serine and threonine each 15 mg per liter, K_2HPO_4 0.2%, NaCl 0.5%, $MgSO_4$ 0.01% and glucose 0.5%. The pH of both media was 6.8 to 6.9. It was tubed in 5 ml amounts.

All tubes of each experiment were inoculated with the same amount of a dilution of a 24-hour culture grown in the ammonium phosphate basal medium with the 4 needed vitamins. Plate counts with dextrose agar indicated that the number of yeast cells in the inoculum in the various experiments was usually from 2,200 to 3,000 per ml of medium. Incubation was at 37°C since this yeast grows well at that temperature. The turbidity resulting from growth was measured in a colorimeter with selected 15 mm test tubes containing 5 ml of medium. In Table I a clear tube of the culture medium is shown by zero and increasing turbidities are shown by higher figures.

Omission of thiamin from the 4 vitamins shown in combination 2 of Table I produced only slight delay in the rate of growth in either basal medium. The omission of pantothenic acid resulted in some delay in growth which, peculiarly, was more marked in the amino acid than in the ammonium phosphate medium. This rather striking difference in the rate of growth in the two basal media, in the absence of pantothenic acid, was observed on repeated tests. It emphasizes the importance of the basal medium in relation to the results obtained in studies of accessory growth factor requirements.

The omission of nicotinamide resulted in complete or almost complete cessation of growth. Evidently nicotinamide is essential under the conditions of these experiments.

Omission of biotin gave rise to interesting results. This compound is needed for prompt growth but slow multiplication may be obtained without it when the other three accessory factors are supplied. The rate of growth in the absence of biotin was quite different in the two basal media; in the ammonium phosphate medium usually 5 to 10

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

TABLE I.
Effect of Certain Vitamins upon Growth of *Torula cremoris*.

Supplement to basal medium	Basal medium: Ammonium phosphate-glucose-salts, Growth by days 1	2	3	7	Basal medium: Amino acids-glucose-salts, Growth by days 1	2	3	7
1. None (control)	0	0	0	0	0	0	0	1
2. Nicotinamide, biotin, Ca pantothenate, and thiamin	22	47	48	56	32	39	42	49
3. (2) minus thiamin	8	44	45	55	21	35	39	47
4. (2) " Ca pantothenate	0	27	40	56	0	0	1	20
5. (2) " nicotinamide	2	3	3	8	4	6	9	9
6. (2) " biotin	0	0	0	7*	3	19	25	28
7. Biotin plus nicotinamide	0	2	30	53	0	0	0	22
8. " " Ca pantothenate	0	0	2	2	0	1	1	3
9. Nicotinamide plus thiamin	0	0	0	2	0	0	0	4

* Heavier growth later.

days were required for attainment of visible growth, while in the amino acid medium growth of the torula was always quite marked by 48 hours.

This speedier growth in the latter instance was found to be due largely to aspartic acid (100 to 200 μg per ml of medium). A similar effect was obtained with synthetic dl-aspartic acid.[1] Other amino acids with the possible exception of glutamic acid did not exert this effect. Further work has shown that the effect of aspartic acid is somewhat more pronounced if the tubes are incubated in an atmosphere of 10% CO_2 in air, though the growth is not as prompt as that caused by biotin either under ordinary atmospheric conditions or in 10% CO_2.

Biotin and nicotinamide together are sufficient to support growth of *Torula cremoris*, though culture development is slower than when thiamin and calcium pantothenate are also supplied. Other combinations of two accessories resulted in either poor growth or no growth. Results with several of the possible combinations are shown in Table I.

Our results with the combination of nicotinamide and thiamin are similar to those of Robbins and Kavanagh[2] who reported that *Torula cremoris* failed to grow in a medium of aspargine, dextrose, ammonium nitrate and inorganic salts when supplemented with either the pyrimidine or thiazole components of thiamin or with thiamin plus nicotinamide.

When added singly each of the 4 accessories failed to support continued growth with but one exception. In the presence of only nicotinamide slow growth was maintained through 10 successive transfers in the amino acid medium but not in the ammonium phosphate medium.

Successive transfers of the torula were also attempted in the ammonium phosphate basal medium with all of the combinations of accessories shown in Table I. Those in which growth could be carried through 10 successive transfers were combinations 2, 3, 4, 6 and 7. Cultures failed to grow either in the first tube or after the first few transplants in numbers 1, 5, 8 and 9.

Experiments were conducted with decreasing amounts of biotin methyl ester in the presence of an excess of nicotinamide, calcium pantothenate and thiamin in the ammonium phosphate basal medium. The results were in harmony with other reports showing the extreme activity of biotin. One millionth μg per ml of medium supported very light growth, 0.00006 μg supported about half maximum growth and 0.002 to 0.003 μg supported approximately maximum development.

Summary. Nicotinamide, biotin, calcium pantothenate, and thiamin must be supplied for prompt and abundant growth of *Torula cremoris* in a basal medium of inorganic salts, glucose and ammonium phosphate

[1] Koser, S. A., Wright, M. H., and Dorfman, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 204.

[2] Robbins, W. J., and Kavanagh, F., *Plant Physiology*, 1938, **13**, 611.

or amino acids. The combination of nicotinamide and biotin supports slow growth and under certain conditions nicotinamide alone may suffice.

Tests with the 4 vitamins singly and in various combinations show that the response to the vitamins depends to some extent upon the basal medium used in the tests. The results emphasize the importance of the composition of the basal medium in studies of vitamin requirements.

14270

Studies on Prothrombin: IV. The Prothrombinopenic Effect of Salicylate in Man.

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Following the demonstration[1] of the quantitative degradation of 3,3′methylenebis(4-hydroxycoumarin) to 2 mols of salicylic acid, Link and his students[2] demonstrated that in the rat salicylic acid induces a prothrombinopenia preventable by natural or synthetic vitamin K.

The purpose of this paper is to report that the fundamental observations made on the rat also apply to man.†

Experimental. The action of sodium salicylate, of acetyl salicylic acid with and without dicumarol (3,3′methylenebis(4-hydroxycoumarin)), and the effect of synthetic vitamin K were studied on individuals representing types in which salicylate therapy is commonly used. Since there was variation in age, nutritional state and diet, the salient facts can best be illustrated by representative cases and with dosages comparable to those used therapeutically.[3]

Twenty-seven adults varying in ages between 24 and 80 years were studied. Eight were apparently normal and ambulatory; 12 were chronically ill patients; 6 were cases of Laennec's cirrhosis of the liver; 1 was a case of acute rheumatic endocarditis.

The method used to estimate the prothrombin level (or activity) included a measure of the prothrombin time of whole and diluted (12.5%) plasma.‡ The rationale and clinical applications have been set forth in previous communications.[4–7] By estimation of 12.5% plasma prothrombin time the initial prolongation has usually been detected between 8 and 24 hours after adequate dosage of salicylate. It is obvious from Fig. 1 that detection of a change in prothrombin time on whole plasma is restricted to a very slight shift in seconds, but with 12.5% plasma a more practical working range is afforded.

Action of Sodium Salicylate. Fig. 2, curve 1 shows the response in a patient who received 8 g of sodium salicylate on 3 consecutive days. Two others receiving the same

* Present address: Central Laboratories, General Foods Corporation, Hoboken, New Jersey.

1 Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

2 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

† This was investigated at the same time by O. O. Meyer at the Wisconsin General Hospital, Madison.

3 Hanzlik, P. J., *Action and Uses of the Salicylates and Cincophen in Medicine*, The Williams and Wilkins Co., Baltimore, 1927.

‡ All estimations were done in duplicate.

4 Shapiro, S., Sherwin, B., Redish, M., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 85.

5 Shapiro, S., Redish, M. H., Campbell, H. A., *Am. J. Med. Sc.*, in press.

6 Shapiro, S., Redish, M. H., Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 12.

7 Shapiro, S., Sherwin, B., and Gordimer, H., *Ann. Surg.*, 1942, **116**, 175.

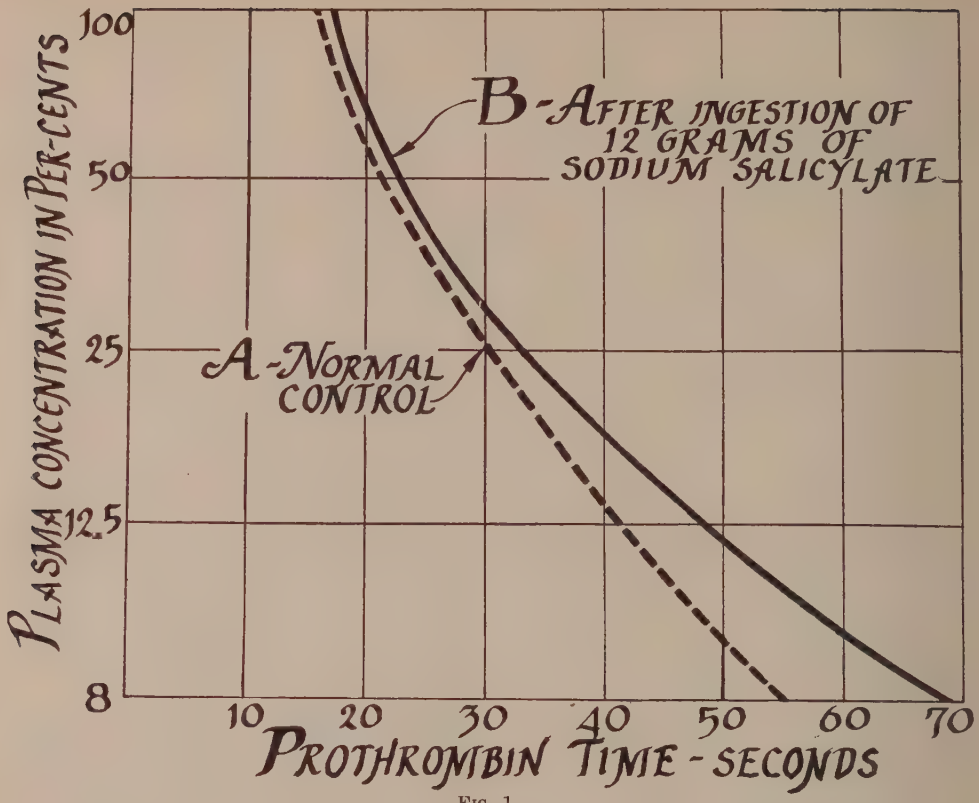

FIG. 1.
Representative dilution curve of normal and sodium salicylate-prothrombinopenic plasma.

dose for one day only showed increases of 6 and 9 seconds and 8 who were given 8 g daily for 2 successive days showed prolongation of 7 to 12 seconds (average 10). Five cases of Laennec's cirrhosis§ exhibited more pronounced prolongation after 16 g in 2 days. Four of these revealed increases between 13 and 23 seconds (average 17) and one yielded an extension from an initial value of 56 to 99 seconds.‖

Two cases in which the drug had to be withdrawn during the first day because of vomiting showed no increase in the prothrombin time. In one case of acute thrombophlebitis which initially revealed a reduction below normal of the difference between whole and 12.5% plasma prothrombin time (hyperprothrombinemia),[4] no alteration in the prothrombin time was detected after 24 g of sodium salicylate had been given in 3 days.

Action of Acetyl Salicylic Acid. Four subjects showed prolongation varying from 5 to 31 seconds in the 12.5% plasma prothrombin time after ingestion of 6 g of the drug in one day. Increases of 8, 12, and 14 seconds after 8 g in one day were found in 3 subjects, and in 1 an extension of 10 seconds after ingesting 16 g in 2 days.

Protective Action of Synthetic Vitamin K. Fig. 2, curve 2, shows the response of an apparently normal man, weighing 70 kg, to 8 g of sodium salicylate on each of 2 successive days, and then the protective action obtained with a very high dosage of synthetic vitamin K (20 mg orally and 2 mg intramuscularly 3

§ All had moderate prothrombinopenia: 12.5% plasma prothrombin time was 1½-2 times the normal value.[5]

‖ These findings are similar to those obtained in cirrhotics with dicumarol.[5]

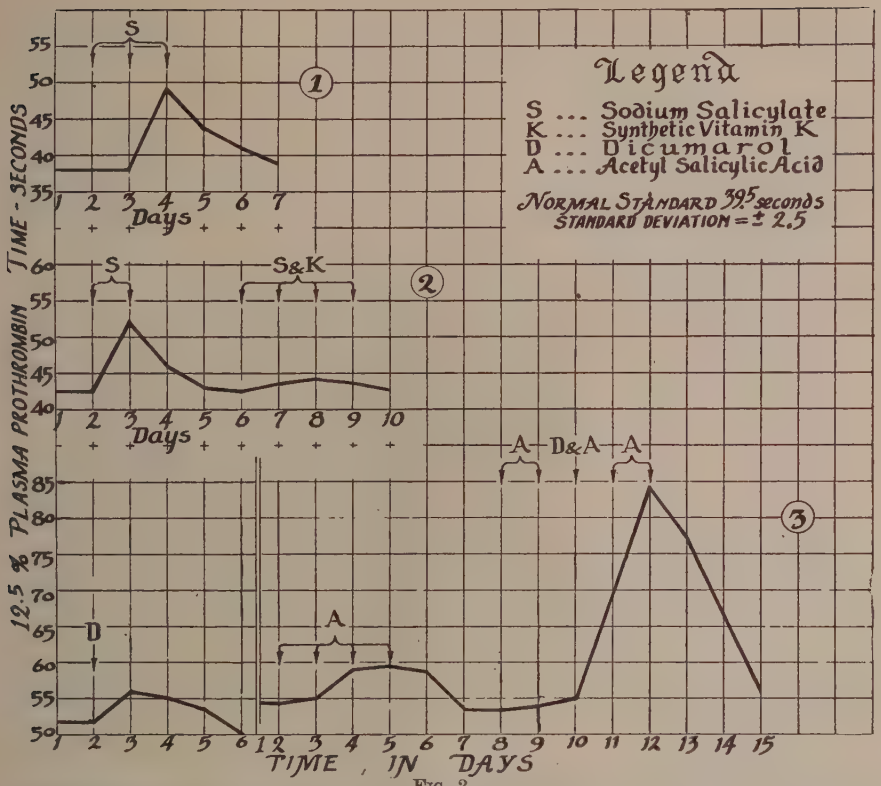

FIG. 2.

Representative effects of salicylate, protective action of synthetic vitamin K, and the combined action of acetyl salicylic acid and dicumarol.

times daily) on 4 successive days, with the same dose of salicylate. Three additional normals and 1 cirrhotic were treated similarly. In the normals the prothrombin time remained substantially the same, while the case of cirrhosis showed approximately the same degree of prolongation as when salicylate alone was given. At the outset these very high levels of synthetic vitamin K were used to insure adequate protection. Subsequent studies indicate that in normals 1 mg synthetic vitamin K intramuscularly can counteract the prothrombinopenia induced by 6 g acetyl salicylic acid given for 1 day.

Combined Effect of Acetyl Salicylic Acid and Dicumarol. Fig. 2, curve 3, illustrates the response in a case of Laennec's cirrhosis with preëxisting prothrombinopenia. The response to a single dose of 50 mg dicumarol is shown first (a comparable increase in a normal subject requires 100-150 mg dicumarol). One week later 4 g acetyl salicylic acid were given for 4 consecutive days. On the seventh day the prothrombin time was at the control level. On the eighth to the twelfth day inclusive 4 g acetyl salicylic acid were given daily. On the tenth day 50 mg dicumarol was also administered. The prothrombin time became prolonged to a maximum of 84 seconds, the control value being restored in 3 days.

The identical procedure was followed in 2 normal subjects. In neither instance did the 50 mg dicumarol or the acetyl salicylic acid alone increase the prothrombin time. Administered together the 2 drugs effected a prolongation in 1 case of 16 and in the other of 18 seconds.

Discussion. The significant prothrombinopenic action of salicylates raises a series

of problems for clinical workers. The diet and nutritional state (vitamin K-prothrombin reserves) of subjects being treated with salicylates merit close attention.[8-12] The fact that these vary in different individuals accounts for the inequalities in response noted above and shown in Fig. 2. This is also true of responses obtained with dicumarol.[5]

Cirrhosis of the liver and pre-existing prothrombinopenia which are known to augment the action of dicumarol[5] exert the same influence when salicylate is administered; both drugs may act through the same enzyme system in the liver.[13-14]

The different salicylates vary in potency as prothrombinopenia-inducing agents. In man as well as in the rat¶ acetyl salicylic acid seems to be more potent than the sodium salt. Additional data on this question as well as minimum effective dosage levels of vitamin K will be reported later.

The complementary action of salicylate and dicumarol may be important in the clinical use of the latter. Their accidental concomitant administration may be the basis for some of the extraordinary responses observed in man.** Both drugs might under certain conditions be given simultaneously to advantage. Work in Link's laboratory¶ (unpublished) indicates that animals can be kept mildly prothrombinopenic without manifestations of bleeding by first giving a moderate dose of dicumarol followed by smaller dicumarol-salicylate dosage.

Based on the fact that the prothrombinopenia can be restored to normal by a preparation of purified prothrombin,[1] fresh blood transfusion and vitamin K should be effective adjuncts in counteracting acute salicylate poisoning. The warning of Sir Arthur Hurst[15] that clinicians should be alert to the possibility of aspirin producing gastric hemorrhage when other known causes of bleeding have been excluded, is well sustained.

Summary and Conclusions. In man as in the rat, salicylate in adequate dosage induces prothrombinopenia which can be prevented by vitamin K compounds, if liver function is adequate. The effect on the prothrombin level (or activity) is detected most readily by the use of 12.5% plasma. The dietary intake particularly of vitamin K appears to play a significant role in determining the extent and duration of the prothrombinopenia. Cirrhosis of the liver and pre-existing prothrombinopenia augment the effect of salicylate. Acetyl salicylic acid appears to be a more potent agent than sodium salicylate. The action of salicylate is apparently identical with that of the anticoagulant dicumarol, but less effective. The two drugs can complement each other. This may be useful in the clinical application of dicumarol and should be realized to avoid excess prothrombinopenia.

This work was conducted in part at the Home of the Daughters of Israel, New York City, through the generous cooperation of Dr. Julius Raab.

Dicumarol was supplied by Dr. K. K. Chen of Eli Lilly & Co.

Synthetic vitamin K (Namaquin) was supplied by the Endo Company.

Miss Frances Kaufman gave technical assistance.

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10 Barnett, H. L., Powers, J. H., Benward, J. H., and Hartman, A. F., *J. Pediat.*, 1942, **21**, 214.

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¶ Personal communication from Professor Link.

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14271

Non-Hematin Iron in Erythrocytes.

S. Granick. (Introduced by L. Michaelis.)

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The structure of ferritin, a crystalline iron-protein compound containing 23% iron, has been discussed in previous papers.[1,2,3] The iron appears to be present in the form of micelles of colloidal iron hydroxide characterized by Fe atoms containing 3 unpaired electrons, and markedly different from various synthetic colloidal iron hydroxides. Crystals containing this iron have been isolated in relatively high yield from spleen, liver and red bone marrow of various species[4] strongly suggesting that this iron arises from hemoglobin decomposition. Since the amount of non-hematin iron in horse spleen is so great (1.8 to 2.0% Fe in dry weight of spleen), it appeared that a histological study of this tissue might reveal the distribution and perhaps the origin of the iron.

1 Granick, S., *J. Biol. Chem.*, 1942, **146**, 451.

2 Granick, S., and Michaelis, L., *J. Biol. Chem.*, 1943, **147**, 91.

3 Michaelis, L., Coryell, Chas., and Granick, L., *J. Biol. Chem.*, 1943, **148**, 463.

4 Granick, S., *J. Biol. Chem.*, in press.

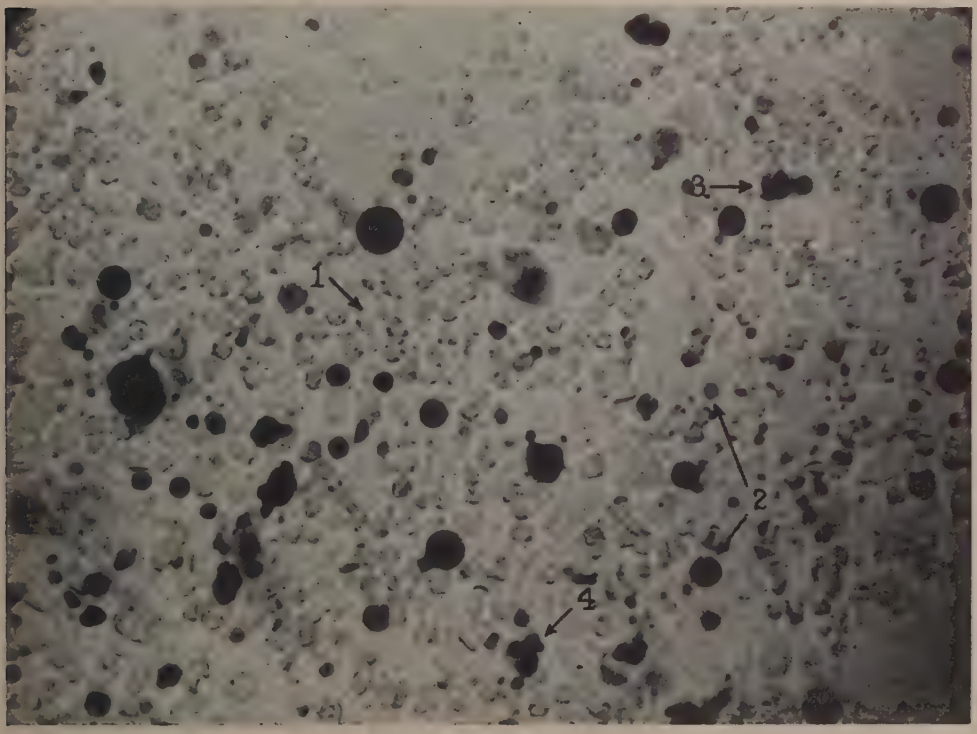

Fig. 1.
H_2S Reaction on Horse Spleen.
1→ Cluster of RBC with black dots.
2→ "Grey" cells.
3→ Hemosiderin granules.
4→ Macrophage containing hemosiderin granules.

A very sensitive test for inorganic iron including the colloidal ferric hydroxide of ferritin is the production of a black insoluble iron sulfide with H_2S. When a piece of horse spleen was teased out into a drop of 1% saline, most of the red blood cells appeared crenated. Cells occasionally appeared which were swollen, had a smooth outline and contained hemoglobin. On the addition of a drop of 1% saline containing H_2S to the tissue, some of the hemosiderin granules became black immediately (3, Fig. 1), thus indicating the presence of iron in them. Other hemosiderin granules remained yellow, indicating that they lacked the non-hematin iron. Some macrophages could be seen to contain large hemosiderin granules which turned black (4, Fig. 1). A number of the crenated erythrocytes developed several very tiny black spots scattered on their membranes (1, Fig. 1). In the presence of H_2S the swollen erythrocytes took on a smooth grey appearance which within a minute became finely granular and darker grey. These latter cells we designate as the "grey cells" (2, Fig. 1).

The "grey" cells have also been observed in horse bone marrow, spleen of guinea pig, spleen of a rabbit and in a human spleen. They have only been observed when the ferritin content appeared to be very high. It was only in the horse spleen that the grey cells could be seen to contain hemoglobin and could therefore be identified as erythrocytes.

In the H_2S reaction the vertebral bone marrow of a 3-year-old horse showed relatively fewer grey cells than did the same animal's spleen. In the marrow the grey cells varied in size from twice the diameter of normal erythrocytes down to 3-5 μ diameter. In a few of the polymorphs the granules also became dark with H_2S.

In the spleens of several guinea pigs with high ferritin content a few grey cells were observed with H_2S; also some black-staining hemosiderin granules in macrophages and some black extracellular granules were observed. In one rabbit spleen a few grey cells were also observed.

In the human material,[4] cases tested with H_2S included No. 10,699 through No. 10,714. Of these cases only those of No. 10,699, monocytic leukemia, and No. 10,709, lymphocytic leukemia, contained much iron in liver and spleen, and these are the 2 cases with the highest ferritin content yet observed. In No. 10,699, the spleen contained a few grey cells per field and a number of tiny pale grey spheres. The liver cells contained numerous heavy black granules. In No. 10,709, the spleen was engorged with erythrocytes among which were present many colorless globules about 1½ times the diameter of the erythrocytes. Only a few of these became pale grey with H_2S. The liver cells were filled with granules but only about 5% of the parenchyma cells contained granules staining grey with H_2S.

Discussion. A plausible explanation for the appearance of the grey cells is that they are degenerating erythrocytes in which the iron of hemoglobin has been set free. It has generally been assumed that red blood cells or their fragments are ingested by macrophages and then broken down. The evidence presented suggests another mechanism; the changes in the erythrocytes may be a function of secretions of the cells lining the sinuses of the spleen. These changes involve increased permeability and fragility and lead to the eventual decomposition of the heme and release of iron without the necessity of phagocytosis by macrophages.

The author desires to express his gratitude to Dr. L. Michaelis and to Dr. G. H. Hogeboom for their suggestions and advice in this investigation.

14272

Extensive Breeding as an Adjunct to Mammary Gland Carcinoma Susceptibility in Mice.

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Susceptibility to carcinoma of the mammary gland in mice of the F_1 generation, following an outcross, is usually determined by the female parent, that is, if a female mouse of a cancer susceptible strain be used in an outcross to a male mouse of a resistant-to-cancer strain the F_1 females develop spontaneous tumors; whereas, if the reverse cross be made, female mice of the F_1 generation do not usually develop spontaneous tumors of mammary tissue. It is Bittner's opinion that the "milk influence" explains this peculiar maternal inheritance of cancer susceptibility. The "forced breeding" principle of Bagg also plays a role in cancer susceptibility but apparently only upon a suitable genetic background.

In the present experiment, consisting of 2 parts, female mice of the JK strain (cancer resistant) were mated to male mice of the C_3H strain (cancer susceptible). The ensuing F_1 individuals were mated either *inter se* or back-crossed to either mice of the original C_3H or JK strains. Mice were continued as breeders for their entire life span, the sexes being kept together. This procedure differs from the "forced breeding" technic of Bagg in that the mice were permitted to nurse their own young, whereas in "forced breeding" the young were discarded within 24 hours of birth. The cyclic changes within the mammary gland are considerably different therefore in the two systems of breeding. Following sexual maturity, the female mice were kept, in this experiment, in an almost continuous reproductive activity—nursing one litter while the next one was developing *in utero*. This regime was greatly aided by maximal nutritional conditions. This reproductive activity was also enhanced by the fact that F_1 individuals show the phenomenon of heterosis, which influences health and vigor of mice as well as of other animals and plants. The mice were fed a diet of Pratt Food Company's Nurishmix, supplemented (a) by fresh washed lettuce twice a week; (b) by fortified or vitamin enriched Bond bread soaked in fresh milk once a week; and (c) by a mixture of 2 parts of oats to one part of wheat twice a week. Water was supplied *ad lib.* Thus conditions for the extensive use of the mice as breeders were satisfied. Seventeen of the 24 F_1 females, with mothers of the JK strain, eventually developed spontaneous carcinoma of the mammary gland at an average of 476.2 days of life. In the reverse cross, 25 of the 30 F_1 females with C_3H mothers developed similar tumors at an average age of 424.6 days. The difference of susceptibility between the 2 F_1 female groups is probably not a significant one.

Thus it would seem that the extensive or excessive use of female mice of the F_1 generation, in this particular cross, between mice of the C_3H and JK strains is an effective way of bringing about spontaneous tumors of mammary tissue. The genetic factor of cancer susceptibility could only have been transmitted through the male. The role of the "milk influence," in this case, is not clear.

In the sequence of events that leads to susceptibility or resistance to spontaneous tumors of mammary origin, there appear to be at present many influences. Among these may be (1) genetic (2) hormonal (3) chemical (4) dietary and (5) normal and abnormal uses such as "forced breeding," and extensive use. As indicated in this paper the role of each of these influences appears to differ considerably under different experimental set-ups. Some of these influences appear to be cumulative in effect, such as the genetic and hormonal; some, such as the chemical and genetic, appear to be supple-

* This experiment has been made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

mentary or alternative. It may be that in this phenomenon of cancer susceptibility there may be still another influence, whose nature is not yet determined, that is also involved in the origin of tumors. Some geneticists believe that this may be in the form of a somatic mutation.

14273

Influence of Spinal Section on Tissue Permeability.*

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The experiments reported here were designed to determine the effect on tissue permeability of transections of the spinal cord at levels varying from the first to the fifth thoracic segment. The spreading of india ink suspensions in the cutaneous connective tissue was used as indicating the degree of connective tissue permeability. Tissue permeability measured in this way is affected by constitutional, nutritional and functional conditions and by the spreading factors (Duran-Reynals)[1] but no influence of the nervous system has been demonstrated. McClean's[2] results, obtained in excised skin, were interpreted as indicating that spreading is independent of vasomotor or other nervous stimuli.

The present study was designed to determine whether or not the nervous system is involved in the regulation of tissue permeability in living animals.

Materials and Methods. Twelve animals (11 rabbits and 1 dog) were used as follows: India ink with saline and india ink with spreading factor† were injected into the skin of the upper and lower part of one side of the animal's body. Twenty-four hours later the extent of spread of the ink spots was recorded by drawing the areas on cellophane and measuring their surfaces in square centimeters with a planimeter.

The spinal cord was then completely transected under local anesthesia at levels varying from the first to the fifth thoracic segment.

Following operation injections of identical amounts of the same mixtures of india ink and saline and of india ink and spreading factor were repeated on the opposite side of the animal's body, care being taken to select injection sites comparable to those used before operation. This was repeated after 24 hours and every subsequent day until all available skin surface was used (usually 3 to 7 days). Twenty-four hours after injection the spread of india ink was traced and measured in the manner indicated above.

For purposes of control 3 animals were laminectomized without spinal cord injury and treated similarly.

All animals were kept in a warm box at 35°C after operation to prevent loss of body heat. Repeated blood pressure readings were taken before and after operation. In all instances the central ear artery was utilized and the blood pressure as determined by oscillometric methods showed constant variations ranging between 65 mm Hg and 95 mm Hg before and after operation.

The spinal animals developed paralysis of the hind legs, urinary bladder and rectal sphincter and in several instances ataxia of

* This work was aided by a grant from the Commonwealth Fund.

† With the technical assistance of Alvin R. Nielsen.

1 Duran-Reynals, F., *Bacteriological Reviews*, 1942, **4**, 197.

2 McClean, D., *J. Path. Bact.*, 1931, **34**, 459.

† India ink (Higgins) was used, diluted in equal parts of saline. All injections contained 0.25 cc of this suspension. The saline injections contained in addition 0.5 cc of physiological NaCl solution. The injections of spreading factor contained 0.5 cc of leech extract in saline 1:10 or extract of rats' testicles in saline 1:10 (0.5 cc). This material was supplied by Dr. Duran-Reynals whose help and advice are deeply appreciated.

the front legs was noted. The latter rabbits were unable to feed and for this reason, food was withheld from the controls. In other respects, the animals remained in good condition throughout the experiment.

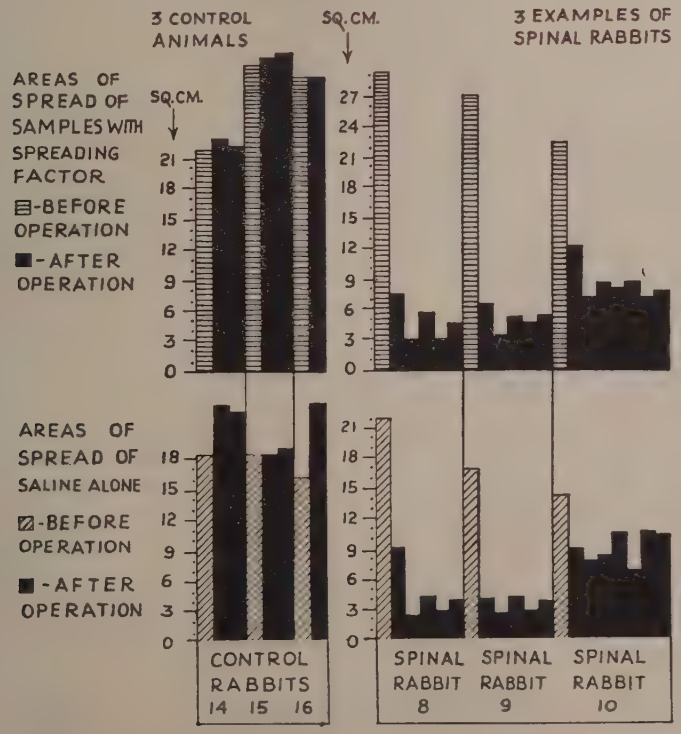
Results. Marked reduction of the spread of india ink was observed in the skin of all spinal animals and occurred in areas above as well as below the level of section of the spinal cord. The reduction in spread was evident in the first sample, injected immediately after operation, and became more pronounced in subsequent injections. After laminectomy, control animals showed either no change or a slight increase in the size of the intradermal ink spots. Chart I presents the results observed in 3 spinal animals (rabbits 8, 9 and 10) and in the control animals (rabbits 14, 15 and 16).

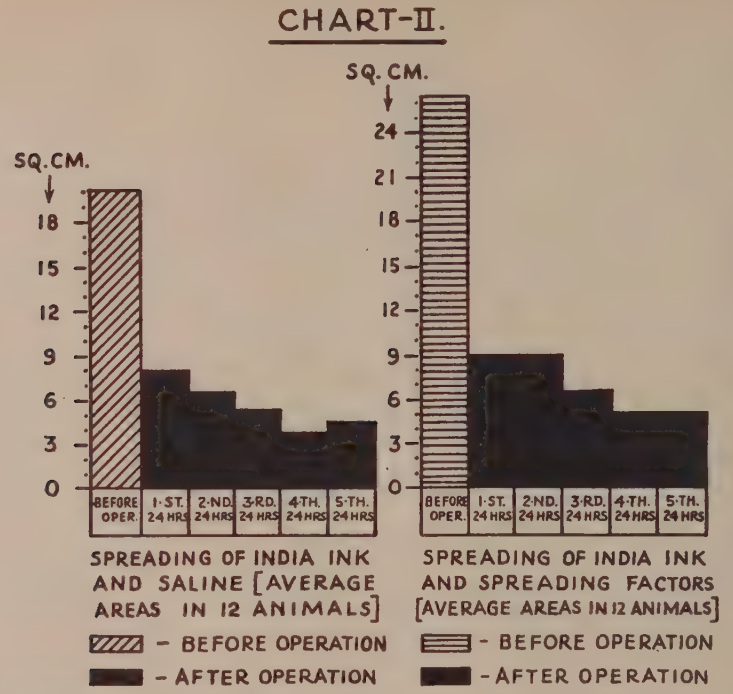
The spread of india ink was reduced in skin areas injected with spreading factor as well as in regions treated with saline. The same proportion in the amount of spread that characterized these substances in intact animals persisted after operation. Pertinent data appear in Chart II in which the columns represent the average amount of spread 24 hours after injection. This was calculated by dividing the total extent of spread in all animals by the number of injection sites.

The activity of the spreading factor can be expressed by the figure obtained by dividing the extent of spread observed in areas injected with the factor by the amount of spread in control areas injected with saline. In the present experiments these values were 1.31 before operation, 1.14 in the first 24 hours following operation, 1.37 in the second 24 hours, 1.26 in the third 24 hours, 1.43 in the fourth 24 hours and 1.25 in the fifth 24 hours. It is clear that the relative activity of the spreading factor was not affected by spinal section.

Comments. The facts presented indicate

CHART - I





that the nervous system takes part in the regulation of connective tissue permeability. The changes observed cannot be due to simple denervation as they occurred above as well as below the level of spinal section. The diminished connective tissue permeability cannot be referred to changes in blood pressure as, contrary to the observations of Wilson and Rigdon,[3] pressure alterations in these experiments were insignificant. It should be emphasized that the observations of these authors[3] applied to the permeability of the capillary endothelium rather than to the permeability of connective tissue and the discrepancies between their results and those presented here illustrate the fact that the permeabilities of these tissues are not identical.[1] The general reduction of permeability following spinal section is in agreement with the work of Lauer[4] who found diminished inflammatory reactions in spinal rabbits above as well as below the spinal section.

Summary of Results. Transection of the spinal cord results in a reduction of connective tissue permeability. The reduction occurs above as well as below the site of spinal section. The reduction is not dependent upon an altered activity of the spreading factor or upon the trauma of operation.

[3] Wilson, H., and Rigdon, R. H., *Arch. Surg.*, 1942, **45**, 416.

[4] Lauer, N., *J. Med. de l'Ucraine*, 1939, **9**, 37.

14274

A Method for Study of Circulation Time Throughout the Vascular System.*

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Introduction. Most methods utilized in the estimation of circulation time determine the circulation time from the arm where a substance is injected to some point in the head where its arrival is detected. These methods determine the rate of flow in a definite and fixed segment of the vascular tree. A method is desirable for the estimation of the circulation time throughout the vascular system. Spier, Wright and Saylor[1] used a solution consisting essentially of magnesium sulfate and calcium gluconate. The arrival of the solution is indicated by a sensation of warmth appearing first in the throat, then in the hands, perineum and feet. These observers and also Kvale and Allen[2] noted a prolongation of circulation time to the feet in patients with peripheral vascular disease. A major objection to this method is that the endpoint is entirely subjective.

The intravenous injection of fluorescein followed by the development of a greenish yellow fluorescence in the palpebral conjunctivae and lips when exposed to ultraviolet light has recently been utilized for the study of circulation time.[3] This method does not permit observations on the circulation in the extremities as normal skin, unlike mucous membrane, does not become fluorescent. The stratum corneum absorbs most of the ultraviolet rays before they reach the capillaries. In an attempt to modify the skin to favor the development of fluorescence, we applied a wheal to the skin by an intradermal injection of various substances. It was found that when a wheal was produced by an intradermal injection of histamine, a brilliant fluorescence appeared in the area about the wheal and in the wheal itself, following an injection of fluorescein.

Methods. The material needed is inexpensive and readily available. It consists of the following: a 20% solution of sodium fluorescein, a 1 to 1000 solution of histamine acid phosphate, a 5 cc syringe with a needle attached (preferably 18 gauge), a tuberculin syringe and needle of small caliber, and a portable source of ultraviolet light. In our present studies we are using a solution of histamine in 0.5% novocaine to prevent the pain which often is associated with the application of the wheal. As the source of ultraviolet light, we found that an argon glow lamp which is supplied with a heat resisting purple filter was adequate and simple to apply.† With the subject lying quietly in the supine position, a wheal is first raised in the skin by an intradermal injection of histamine acid phosphate. The size of the wheal does not appear to be of importance, but it was usually about ½ cm in diameter. In the present study, the wheal was made in the skin of the middle portion of the leg and in a smaller group of subjects, in the anterior aspect of the forearm. The needle of the 5 cc syringe, containing 2 cc of the sodium fluorescein is then inserted into the antecubital vein. The room is darkened and after about 30 seconds the sodium fluorescein is injected rapidly. The ultraviolet light is directed to one of the eyes of the subject and a stop-watch records the time of the appearance of the fluorescence in the palpebral

* Aided by the Lucille A. Metzger Fund for Medical Research.

1 Spier, L. S., Wright, I. S., and Saylor, L., *Am. Heart J.*, 1936, **12**, 511.

2 Kvale, W. F., and Allen, E. V., *Am. Heart J.*, 1939, **18**, 519.

3 Fishback, D. B., Guttman, S. A., and Abramson, E. B., *Am. J. Med. Sc.*, 1942, **203**, 535; Lange, K., and Boyd, L. J., *M. Clin. N. Am.*, 1942, **26**, 943.

† This consists of a standard 2-watt argon glow lamp enclosed in a small reflecting housing. This unit is readily available and built by the Stroblite Company of New York.

TABLE I.
Circulation Time in 80 Subjects.

	Time in sec	
	Arm to conjunctiva	Arm to leg
Group 1.		
Normal individuals (32)	15	20.1
Group 2.		
Individuals above 50 years of age, no evidence of vascular disease (28)	15.1	24.1
Group 3.		
Individuals with *arteriosclerosis obliterans* (20)	15.5	32.9

conjunctiva. The ultraviolet light is then directed to the wheals in the skin and the time of appearance of fluorescence recorded. When several wheals are applied, it is necessary for one observer to inject the fluorescein, while another notes the endpoints at the various sites, recording the time of appearance of fluorescence with the stop-watch.

Results and Discussion. This procedure was carried out in 80 subjects. They were divided into 3 groups. Group 1 includes 32 individuals, all below the age of 50 and free from any evidence of peripheral vascular disease. Group 2 consists of 28 subjects above the age of 50, but with no definite signs of disease of the peripheral vessels. Group 3 includes 20 patients who had definite evidence of vascular disease in the lower extremities. The type of vascular involvement in each instance could be classified as *arteriosclerosis obliterans*. In each of the 80 subjects, the circulation time was recorded from the arm to the conjunctiva and from the arm to a wheal in the leg (arm to leg time). The results are summarized in Table I. In 20 instances, coincident circulation times were recorded from the arm to the conjunctiva and from the arm to a wheal in the opposite arm (arm to arm time). By subtracting the "arm to conjunctiva" time from "arm to leg" and "arm to arm" times, one may obtain a reasonable approximation of the rate of flow in the arterial segment to the leg and arm. It will be noted from Table I that the average circulation time from "conjunctiva to leg" in normals is 5.1 seconds, in the group of elderly subjects 9 seconds, and in the *arteriosclerosis obliterans* group 17.4 seconds. Spier and his associates[1] found the average time from "throat to foot" in normals to be about 14 seconds, while Kvale and Allen[2] report this interval to average about 20 seconds. While our results show the "conjunctiva to arm" time in normals to vary from 1 to 3 seconds, the average reported by Kvale and Allen for "throat to hand" time is from 9 to 10 seconds. The exact explanation for the differences in results is not clear. It should be stated as a possible explanation that any method in which a sensory reaction is used as the end point may include a latent period during which the perception of the sensation develops. It is quite possible that, due to a lower reactivity of the sensory endings in the extremities, a higher concentration of the magnesium-calcium solution must develop at the site of stimulation in order to call forth a response. Thus the time interval observed by this method between the response in the throat and the reaction in the extremities may include not only the circulation time between these points, but also the time required for the development of the sensory response at the end point.

As compared with the calcium-magnesium method, the present procedure shows a sharper differentiation between normal individuals and those with peripheral vascular disease. As compared with a "conjunctiva to leg" time of 5.1 seconds in normals and 17.4 seconds in *arteriosclerosis obliterans* by our method, Kvale and Allen[2] report an average "throat to foot" time of 20.3 seconds in normals and 26 seconds in *arteriosclerosis obliterans*.

Summary. An objective method is described for the study of circulation time throughout the vascular system. The results of the application of this method in normal subjects and in individuals with peripheral vascular disease is reported.

14275 P

Experimental Lathyrism in the White Rat.*

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Lathyrism, associated with the consumption of considerable amounts of certain species of legumes of the genus *Lathyrus*, has been a common disease of man in India, northern Africa, and occasionally in other countries.[1] Lathyrism has also been observed to occur in domestic animals and has been produced experimentally in laboratory animals. The outstanding symptoms are muscular weakness and paralysis of the extremities.

Neither Zagami,[2] McCarrison,[3] nor Visco[4] was able to produce experimental lathyrism (*Lathyrus sativus*, L., and *Lathyrus cicera*) in white rats, although impaired growth and failure of normal organ development, particularly of the reproductive and skeletal systems, were observed. Geiger and coworkers[5] produced lathyrism in rats by feeding diets which contained *Lathyrus odoratus*, the flowering sweet pea, at levels of 80, 50, and 25% of the diet. Characteristic symptoms were lameness, paralysis, and curvature of the spine and sternum.

Our experiments were carried out with finely ground meal (20 mesh sieve) prepared from decorticated sweet peas (*Lathyrus odoratus*) and as a control, with similar meal from the edible split pea of commerce† (*Pisum sativum*, subspecies *arvense*). These were incorporated into the diets of young white rats as follows: pea meal, 50%; casein (Labco, vitamin-free), 10%; corn starch, 27%; sucrose, 5%; salt mixture (modified Osborne-Mendel), 4%; cod liver oil, 2%; corn oil, 2%. Each rat received approximately 200 mg of a dried yeast tablet (Mead Johnson and Co.) daily. The casein was added to the diet to insure a satisfactory protein content, since the proteins of many legumes are known to be inadequate if fed as the sole source of protein. The total protein of the mixed diet was calculated to be 26% (16% derived from the peas).

Young white rats, 50-80 g in weight, in litter units, were paired as to sex and weight. The paired feeding method was employed; one rat of each pair received the sweet pea diet *ad libitum*, while the intake of the paired litter mate fed the edible pea diet was limited by the food intake of the mate fed sweet peas.

In the first series in which 9 pairs were fed, all of the rats fed the *Lathyrus* pea diet showed definite symptoms of lathyrism (incontinence, lameness, paralysis of limbs, spinal curvature of the thoracic region, etc.), symptoms being observed in from 2 to 7½ weeks. No control (edible pea diet) animals showed any abnormalities during the period of observation. In addition, 2 pairs received extra casein to give a total dietary protein content of approximately 41% (25% of casein), the extra casein replacing an equivalent amount of starch. The presence of larger amounts of a superior

* Aided by a grant from the Faculty Research Fund of the Horace H. Rackham School of Graduate Studies, University of Michigan.

1 Stockman, R., *J. Pharm. and Exp. Therap.*, 1929, **37**, 43.

2 Zagami, V., *Atti della Reale Accademia Nazionale dei Lincei*, Ser. 6, Rendiconti 1931, **14**, 218; abstracted in *Chem. Abs.*, 1932, **26**, 3823.

3 McCarrison, R., *Ind. J. Med. Res.*, 1928, **15**, 797; McCarrison, R., and Krishnan, B. G., *Ibid.*, 1934, **22**, 65.

4 Visco, S., *Arch. farmacol. sper.*, 1923, **35**, 39; **37**, 1; **47**, 269; abstracted in *Chem. Abs.*, 1923, **17**, 1983; 1924, **18**, 1345, 2544.

5 Geiger, B. J., Steenbock, H., and Parsons, H. T., *J. Nutrition*, 1933, **6**, 427.

† We are indebted to Mr. Raymond H. Coulter, of the Ferry-Morse Seed Company of Detroit, Michigan, for the sweet pea seed generously placed at our disposal, and to Mr. Floyd Trail, of the Washburn-Wilson Seed Company, of Moscow, Idaho, who supplied the edible split peas.

animal protein in the diet did not prevent or delay the onset of lathyrism, since characteristic symptoms were observed in 3½ to 5 weeks.

In a second series the effect of cold water extracts of the peas was studied. *Lathyrus* peas were extracted overnight at 0° with distilled water, the fluid decanted and extraction repeated 3 times. The combined extracts were evaporated to a thick syrup *in vacuo*. Edible split peas were similarly extracted. To the split pea residue was added the extract of the *Lathyrus* peas and to the *Lathyrus* residue, the split pea extract. The combined extract and residue in each case were dried in thin layers at 80°, ground finely and incorporated into the diets, as described above. All of the rats receiving the *Lathyrus* extract-split pea residue developed acute lathyrism in from 2½ to 4½ weeks, while none of the rats receiving the split pea extract-*Lathyrus* pea residue diet showed any symptoms in 7 to 8 weeks. It thus appears that the toxic substance is readily extracted from *Lathyrus* meal by cold water and that the extracted residue is relatively non-toxic. This is in accord with the findings of Geiger,[5] who extracted peas with boiling water.

Pathological studies of the bones made with the cooperation of Professor Carl V. Weller revealed changes in the long bones of the leg similar to those observed in acute scurvy. The rat is believed to be able to synthesize ascorbic acid and does not require the preformed dietary vitamin. It was believed that the toxic principle of *Lathyrus odoratus* might either affect the synthesis of ascorbic acid or in some way increase the requirement beyond the capacity of synthesis of the organism. In a third series, rats fed the sweet pea diet were paired, one animal of each pair receiving in addition 12 mg of ascorbic acid. All of the animals showed symptoms of acute lathyrism within the usual period of time. No preventive effect of the ascorbic acid could be demonstrated.

Further experiments designed to determine the nature of the toxic principle are in progress.

14276 P

Role of the Premotor Cortex in Human Motor Activity.

JEFF MINCKLER AND ROLAND M. KLEMME. (Introduced by A. Kuntz.)

From the Department of Anatomy and the Section of Neurosurgery, St. Louis University School of Medicine.

The collateral systems (thalamus, basal ganglia, cerebellum, and premotor cortex) which play such a prominent role in human motor activity are particularly refractory to experimental manipulation, due to the fact that complex motor dysfunction is peculiarly human and has no certain counterpart in lower mammals. It is desirable, therefore, to employ human material for information relative to the motor complex. This report serves to clarify certain points concerning the anatomy and function of the human premotor region.

A patient (aged 38 years) was subjected to bilateral (2-stage) premotor cortical excision (Klemme)[1] for the relief of generalized tremor in both upper extremities, moderate tremor and rigidity of the left lower extremity, and tremor of the tongue. The right side was operated first with relief of the contralateral tremor. The left side was operated 9 months later, the patient expiring on the seventh post-operative day. At no time was there loss of volitional movement, the residua being generalized weakness, more marked on the left, and inability to do finely coördinated movements with the left hand. Degeneration studies with the Swank-Daven-

[1] Klemme, R. M., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **21**, 596.

port and Weigert technics, which showed the effects of the right (old) lesion only, revealed the following relationships. The efferent bundles from the excised region follow two distinct pathways: (1) commissural fibers which pass to the opposite premotor cortex via the corpus callosum; and (2) projection fibers which aggregate on the lateral aspect of the superior part of the lateral ventricle, pass through the posterior limb of the internal capsule and continue through the lateral aspect of the medial one-third of the basis pedunculi, the medial aspect of the corticospinal field in the pons, diffusely through the pyramid, and enter the spinal cord in the medial aspect of the homolateral anterior white column. Insofar as the myelin stain permits, terminal relationships are indicated to thalamus, substantia nigra, brain stem nuclei, and the anterior horn of opposite side in the spinal cord.

The influence of the premotor bundle on involuntary movements incident to diseases of the basal ganglia has been amply demonstrated by alleviation of the movements through surgical interruption of the pathway. Success has attended operations or pathology in which: (1) the premotor area is excised (Klemme,[1] Bucy[2]), (2) parts of the basal ganglia and considerable subcortical tissue is destroyed (Meyers),[3] (3) the internal capsule is damaged by hemorrhage (Wilson),[4] (4) the pyramid is sectioned (Putnam),[5] and (5) the anterior white column in the spinal cord is cut (Putnam).[5] In all these procedures the bundle just described would be interrupted.

This schema of premotor relationships differs from Bucy's[6] parapyramidal system in being a continuous pathway from cortex to spinal cord and is in agreement with Kennard's[7] findings in the monkey. While the differences in the pathway do not make the mechanisms involved incompatible with those outlined by Bucy, we wish to call attention to the relationships of the premotor bundle at the final-common-path cell as an additional factor in the mechanism. Of approximately 4000 terminals ending in relationship to the largest anterior horn cells (Minckler)[8] only 10% are derived from cortical levels. In the light of recent information on synaptic transfer it would seem doubtful that these few terminals could, alone, initiate a discharge at the final-common-path. It is more probable that additional terminals come into play in governing the final discharge. While an irregularity in discharge manifested by involuntary movements may have its ultimate inception in distorted cerebral function (Bucy),[6] the immediate muscle activity is derived through the final-common-path. It would seem, then, that the interrelationships at this point must be included in any explanation of the mechanics of movement.

Summary. The premotor cortex of man gives rise to bundles passing (1) to the premotor cortex of the opposite side and (2) through the internal capsule to thalamus, brain stem nuclei, and spinal cord. Interruption of this pathway alleviates the involuntary movements incident to disease of the basal ganglia. The interrelationships of terminals at the final-common-path are considered important in explaining the mechanism of these movements.

2 Bucy, P. C., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **21**, 551.

3 Meyers, R., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **21**, 602.

4 Wilson, S. A. K., *Modern Problems in Neurology*, William Wood & Co., N. Y., 1929.

5 Putnam, T. J., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **21**, 666.

6 Bucy, P. C., *J. Neuropath. and Exp. Neurol.*, 1942, **1**, 224.

7 Kennard, M. A., *Arch. Neurol. and Psychiat.*, 1935, **33**, 698.

8 Minckler, J., *Science*, 1942, **96**, 117.

SECRETARY'S REPORT

April 1, 1942-March 31, 1943

At the request of the United States Coordinator of Transportation our Society did not hold annual meetings of the Council, the National Membership Committee, the Board of Editors, and the Section Secretaries. The emergency committee authorized by the Council met to consider certain problems arising from the war emergency. Wherever possible the business of the Council was transacted by mail.

U. S. Board of Censors. It is now necessary to submit galley proof of each number of the PROCEEDINGS to the U. S. Board of Censors. If in their judgment there are articles which may be of use to the enemy, no export license is issued, and that number of the PROCEEDINGS may not be mailed to certain South American and certain neutral European countries. So far only two numbers of the PROCEEDINGS have not received this license. We have notified our South American subscribers and members of this ruling, and have asked them whether they wish us to hold such copies for the duration, send them copies with certain articles removed, or return check for their subscription or dues.

Election of Officers. President W. deB. MacNider appointed as tellers, Doctors Cattell, Charipper, and Root. The tellers report the following officers elected:

President—A. J. Carlson
Vice-President—A. B. Hastings
Secretary-Treasurer—A. J. Goldforb
Councillors at Large—E. A. Doisy
C. J. Wiggers

New Members. The National Membership Committee includes one member from each of the nine largest Sections of the Society. They are Doctors E. F. Adolph, R. N. Bieter, C. A. Dragstedt, H. Goldblatt, J. A. Greene, I. Greenwald, W. C. Hess, M. Kleiber and A. Kuntz.

It is not generally known how much labor, meticulous care, and fair evaluation have been exercised by this Committee. Eighty-nine applicants were approved. The Council subsequently concurred. The applicants elected to membership are:

J. C. Abels
A. M. Ambrose
R. K. Anderson
C. D. Aring
I. L. Baldwin
Philip Bard
E. F. Beach
F. A. Beach
W. D. Block
Siegbert Bornstein
F. G. Brazda
B. B. Brodie
R. H. Broh-Kahn
F. A. Brown, Jr.

S. C. Bukantz
C. G. Burn
E. G. Butler
G. F. Cartland
J. Casals
N. F. Conant
E. S. Cook
F. S. Daft
Henrik Dam
N. A. David
M. E. DeBakey
H. G. Day
A. L. Dounce
D. P. Earle, Jr.
D. W. Fassett
W. H. Feinstone
C. L. Fox, Jr.
W. H. Gates
L. P. Gebhardt
C. E. Georgi
E. D. Goldsmith
Horace Goldie
F. B. Gordon
G. M. Guest
L. R. Hae
P. L. Harris
Nellie Halliday
R. C. Ingraham
M. W. Jennison
William Kaufmann
N. C. Klendshoj
F. L. Kozelka
Maurice Landy
G. L. Laqueur
Milton Levine
H. D. Lightbody
Bernard Lustig
E. W. McChesney
W. H. McShan
H. W. Magoun
John Maier
Abraham Mazur
Norton Nelson
H. W. Nilson
H. S. Olcott
M. J. Oppenheimer
O. S. Orth
A. Packchanian
A. J. Patek, Jr.
S. A. Peoples
N. M. Phatak
T. S. Potter
W. O. Reinhardt
J. I. Routh
W. D. Salmon
David Scherf
Austin E. Smith
Floyd R. Smith
John R. Smith
A. E. Sobel
Frederick Steigmann
F. M. Strong
Y. Subbarow
W. H. Summerson
W. H. Taliaferro
R. S. Teague
Cesare Tedeschi
Albert Tyler
K. R. W. Unna
J. F. Volker
K. G. Wakim
C. K. Weichert
C. F. Winchester
J. G. Wooley
D. W. Woolley

As much time is required for transmission of applications to all members of the Committee and to the Council, it is necessary that all applications for membership be received in this office by December 15.

Members Emeritus. A member who has retired from active duty in his institution may on request be elected to emeritus membership. Such member does not pay dues and does not receive the PROCEEDINGS. The following were elected emeritus members: J. F. Anderson, W. B. Cannon, E. G. Conklin, B. M. Duggar, C. W. Duval, A. I. Kendall, Adolf Meyer, H. B. Torrey.

Resignations. Fourteen members applied for resignation. The Council concurred with regret.

Members in Arrears. The Council most reluctantly dropped six members for arrears.

Deaths. The Council records with deep regret the death of the following members: E. Allen, E. Andrews, W. H. Brown, G. Calkins, J. F. Daniel, H. R. Geyelin, R. A. Gortner, A. D. Hirschfelder, P. S. McKibben, W. L. Niles, J. A. F. Pfeiffer, I. Pineussen, S. W. Ranson, M. Sandberg, F. H. Swett, D. C. Walton, K. Landsteiner.

Peiping Section. Due to war conditions the members of the Peiping Section were scattered.

Finances. For the fiscal year ending March 31, 1943, there was a surplus of $3,200. Nearly all of this was invested in United States Bonds. It was difficult to anticipate, at the beginning of the fiscal year, the number of members to be sent to war areas. These may on request have their dues cancelled. We could not estimate loss of subscriptions from war zones. In December, 1942, it was calculated that a large surplus would occur. Hence costs to authors were reduced. Authors who had published prior to this received refunds for the difference in the amount charged and the new reduced charges. The Council has authorized further reduction in costs of excess space for the year beginning April 1, 1943, from 40% to 24% of our cost.

Trustees. The Society is greatly indebted to Doctors R. A. Lambert and C. D. Leake, Trustees, who have with their advisors, so conscientiously guarded our investments in these difficult times. Special thanks are due to Mr. H. G. Friedman of General Investors, Inc., and Mr. Edward Robinson of the Rockefeller Foundation, who have given so freely of their time and knowledge in protecting our investments. Mr. Leon Leighton has continued to aid us in all legal phases of our mortgage investments. Needless to say, these services have been given without compensation.

Indices of Proceedings. The Society is again indebted to Dr. Emil Baumann for the excellent indices in each volume of the PROCEEDINGS.

Editors. It should perhaps be recalled that there are two to five editors in each major field. The editors may, and often do, consult with specialists in their region. At least three editors must be in substantial agreement before a manuscript is rejected, or the author asked to revise it radically. A manuscript may be referred to editors once, twice, three times or more. The lot of the Editors is an onerous one. The Society owes a great debt of gratitude to the Editors, for their loyal, devoted, and extraordinarily fine work.

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: H. D. Green. Secretary: R. W. Heinle. Members: 46.
Meetings: Western Reserve University, November 13, 1942
December 11, 1942
January 8, 1943
February 12, 1943
City Hospital, March 10, 1943
Western Reserve University, April 9, 1943

District of Columbia

Chairman: H. M. Dyer. Secretary: J. H. Roe. Members: 69.
Meetings: George Washington University, December 3, 1942
February 4, 1943
May 6, 1943

Illinois

Chairman: E. S. G. Barron. Secretary: L. V. Domm. Members: 143.
Meetings: University of Chicago, October 27, 1942
January 26, 1943
March 9, 1943

Iowa

Chairman: R. B. Gibson. Secretary: C. A. Winter. Members: 39.
Meetings: State University of Iowa, November 25, 1942
February 11, 1943
April 29, 1943
May 26, 1943

Minnesota

Chairman: F. H. Scott. Secretary: F. H. Scott. Members: 54.
Meetings: University of Minnesota, November 18, 1942
February 17, 1943

Missouri

Chairman: A. S. Gilson, Jr. Secretary: L. R. Jones. Members: 57.
Meetings: Washington University, May 12, 1943

New York

Chairman: M. Heidelberger. Secretary: W. S. Root. Members: 482.
Meetings: New York Academy of Medicine, December 28, 1942
March 17, 1943

Pacific Coast

Chairman: F. W. Weymouth. Secretary: M. Kleiber. Members: 103.
Meetings: University of California, October 3, 1942
Stanford University, December 2, 1942
University of California, January 20, 1943
April 7, 1943

Peiping, China

Chairman: A. B. Fortuyn. Secretary: F. T. Chu. Members: 30.

Rocky Mountain

Chairman: A. H. Washburn. Secretary: B. B. Longwell. Members: 23.
Meetings: University of Colorado, November 27, 1942
March 5, 1943
Denver, Colo., May 26, 1943

Southern

Chairman: W. A. Sodeman. Secretary: K. L. Burdon. Members: 46.
Meetings: Tulane University, December 4, 1942

Southern California

Chairman: C. A. G. Wiersma. Secretary: B. Krichesky. Members: 47.
Meetings: University of Southern California, October 22, 1942
California Institute of Technology, January 21, 1943
University of California, Los Angeles, May 6, 1943

Western New York

Chairman: R. K. Brewer. Secretary: A. H. Hegnauer. Members: 65.
Meetings: University of Rochester, October 17, 1943

Wisconsin

Chairman: A. L. Tatum. Secretary: L. E. Casida. Members: 44.
Meetings: University of Wisconsin, January 15, 1943
February 17, 1943
March 17, 1943

MEMBERSHIP

Members, March 31, 1942	1625	
Elected during year	127	
Total		1752
Resignations	21	
Arrears	4	
Deaths	17	
		42
Total Membership, March 31, 1943		1710

Membership:	1933	1943
	1146	1710

Subscriptions, March 31, 1943 636

TREASURER'S REPORT

April 1, 1942-April 1, 1943

Balance on hand, April 1, 1942		$ 9,722.95
Income		
Dues	$ 6,723.67	
Reprints	3,026.01	
Space	2,370.71	
Cuts	506.64	
Changes	43.74	
Subscriptions	6,221.36	
Back Numbers	180.50	
Interest from special account	1.97	
Miscellaneous	3.36	
		$19,077.96
Total Cash Available, April 1, 1942-43		$28,800.91
Disbursements		
Printing	$ 8,440.38	
Reprints	2,844.86	
Cuts	613.13	
		$11,898.37
Office Supplies, Telephone, Postage	$ 913.13	
Salary	2,405.70	
Storage and Insurance	71.13	
Refunds	241.25	
Miscellaneous	392.39	
		$ 4,023.60
		$15,921.97
Transfer of Endowment Fund from Corn Exchange Bank		749.29
Purchase of Government Bonds from Surplus 1941-42		3,000.00
		$19,671.26
Cash Balance, April 1, 1943		9,129.65
		$28,800.91

Income (net)	$19,077.96
Disbursements (net)	15,921.97
Surplus	$ 3,155.99

Accounts receivable—$817.12
Accounts payable—$20.80 (Victory Tax—1/1/43 - 3/31/43).

FUNDS

Endowment Fund

April 1, 1942	$19,135.57	
Interest to April 1, 1943	666.39	
		$19,801.96
Invested in		
New York Title and Mortgage Co.	$ 5,580.00	
Title Guarantee and Trust Co.	1,905.99	
Lawyers Mortgage Co.	1,478.32	
Bowery Savings Bank	2,914.03	
U. S. Savings Bonds	5,000.00	
Industrial Bonds	2,923.62	
		$19,801.96

Surplus Fund

April 1, 1942	$11,781.49	
Interest to April 1, 1943	437.54	
Transfer of 1941-42 surplus	3,000.00	
		$15,219.03
Invested in		
Title Guarantee and Trust Co.	$ 2,773.35	
Harlem Savings Bank	1,596.49	
U. S. Savings Bonds	4,975.00	
Industrial Bonds	5,874.19	
		$15,219.03

Life Membership Fund

Invested in Railroad Federal Savings and Loan Assn.	$ 75.00

Auditors' Report

We have examined the books of the Society and believe the records of the financial transactions to be accurately set forth in the Treasurer's report.

(Signed) R. K. CANNAN
S. Z. LEVINE
P. K. OLITSKY

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